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Post-streptococcal autoimmune neuropsychiatric disease: clinical spectrum and identification of brain auto-antigens.

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To Mum and Dad

TABLE OF CONTENTS

Lists of Tables and Figures

Chapter 1. Introduction. Clinical features.

Tables

Figures

Chapter 2. Methods. Clinical study.

Tables

Chapter 3. Results- The clinical spectrum of post-streptococcal neuropsychiatric disease

Tables

Figures

Chapter 4. Introduction- immunopathogenesis of post-streptococcal neuropsychiatric disease.

Tables.

Figures

Chapter 5. Protein purification and immunology methods

Figures

Chapter 6. Proteomic identification of the 45 and 98 kDa autoantigens

Tables

Figures

Chapter 7. Proteomic identification of the 40 kDa autoantigen

Tables

Figures

Chapter 8. Proteomic idenitification of the 60 kDa autoantigen

Tables

Figures

Chapter 9. In vitro pathogenic effects of anti-neuronal antibodies on neuronal function

Tables

Figures

Chapter 10. Discussion of clinical and immunological thesis findings

Tables

Figures

Chapter 11. Conclusions

Figures

Abstract

Group A beta haemolytic Streptococcus (GAS) is a bacteria that causes a range of invasive and autoimmune complications in humans. It is also the commonest single cause of tonsillitis in children. Occasionally, in predisposed individuals, GAS can induce an immune mediated brain syndrome with particular vulnerability of the basal ganglia. This results in extrapyramidal movement disorders (classically chorea) and psychiatric disease. Recently the clinical spectrum of disease associated with GAS has been expanded to include motor tics. The clinical aim of this thesis was to define the clinical spectrum of CNS disease associated with GAS in a tertiary referral sample. I found that a broader range of extrapyramidal movements (chorea, tics, dystonia and Parkinsonism) and psychiatric disease (obsessive-compulsive disorder, anxiety, depression and attention deficit hyperactivity disorder) occurred in patients with post-streptococcal autoimmune neuropsychiatric disease.

The proposed mechanism of brain injury is cross-reactivity of the immune response between GAS and brain antigens (molecular mimicry). A number of investigators have previously demonstrated auto-antibodies that bind to brain auto-antigens in the serum of post-streptococcal neuropsychiatric patients. Using protein purification strategies (2-dimensional electrophoresis, ion exchange and hydrophobic interaction chromatography followed by mass spectrometry), I identified the brain antigens as neuronal isoforms of the glycolytic enzymes enolase, pyruvate kinase and aldolase. These findings were confirmed with commercial antigens, recombinant human antigens and commercial antibodies. These enzymes are present in the neuronal cytoplasm, but also on the membrane surface where they have a number of functions. Collaborative investigation into the effects of anti-neuronal glycolytic enzyme

antibodies on neurones in vitro demonstrated increased neuronal apoptosis compared to controls. In addition, the enzymes are present on the membrane surface of GAS, therefore representing a potential example of molecular mimicry.

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Chapter 1. Introduction- clinical phenomenology of post-streptococcal neuropsychiatric disease

1.1.Aims of the introduction

The aims of the introduction are as follows:

1. To review the clinical spectrum of post-streptococcal neuropsychiatric syndromes.
2. To review the current understanding of the pathogenesis of post-streptococcal neuropsychiatric syndromes.
3. To establish clear aims for the clinical and immunological aspects of this thesis.

1.2.Overview of post-infectious neurological syndromes

Human beings live on this planet in a complex environment. We are constantly under attack by a broad range of infectious and toxic insults. Our immune system is responsible for protecting us against infection. However, occasionally the host immune system can over-respond to the infectious precipitant and result in immune attack against itself, a concept known as post-infectious autoimmunity. The term 'post-infectious' infers that the infectious precipitant has induced an aberrant immune response in the host that then attacks its own body. Infections have been proposed in a broad spectrum of immune-mediated neurological disease, and examples are summarised in the table below (Table 1.a):

Table 1.a. Recognised post-infectious CNS syndromes

Proposed infectious precipitant	Recognised neurological disorder	Predominant brain region involvement
Group A Streptococcus	Sydenham's chorea	Basal ganglia
Varicella Zoster virus	Post-varicella ataxia	Cerebellum
Multiple viruses/bacteria	Acute disseminated encephalomyelitis, optic neuritis, transverse myelitis	White matter

The mechanism by which the infectious agent interacts with the immune system and results in neurological disease is likely to be complex and varies from infectious agent to infectious agent. The neurological syndromes associated with Group A Streptococcus will be the subjects of this thesis.

1.3.Group A streptococcus

1.3.1.Group A streptococcus and humans

Group A streptococcus (GAS) (*Streptococcus pyogenes*) is a common pathogen in humans and one of the most versatile pathogens. It causes common infections (pharyngitis, impetigo, cellulitis, scarlet fever) but also severe life-threatening disorders (puerperal sepsis, necrotising fasciitis, myositis, toxic shock syndrome and CNS infections). The case fatality of these severe complications ranges from 20-45% (Bisno et al., 2003). In addition, there are 2 classic non-supporative 'autoimmune' complications: acute rheumatic fever and acute post-streptococcal glomerulonephritis. Within the rheumatic fever spectrum is the CNS complication, Sydenham's chorea.

The other major criteria of rheumatic fever include carditis, polyarteritis, erythema marginatum and subcutaneous nodules (Special writing group of the committee on rheumatic fever, 1992).

The recent advances in molecular biology have improved our understanding of GAS virulence. Virulence has been attributed to the ability of an organism to impair phagocytosis and the ability to adhere to epithelial cells. Virulence factors of GAS are listed in table 1.b.

Table 1.b. Proposed virulence factors of GAS bacteria (Bisno et al., 2003).

Virulence factor (known or postulated)	GAS CONSTITUENT
Anti-phagocytic	• M protein • M-protein like (Mrp and Enn) • Hyaluronic acid capsule • C5a peptidase
Adherence to epithelial cells (adhesions)	• Lipoteichoic acid • Fibronectin binding proteins • M protein • Hyaluronic acid capsule
Internalisation	• M protein • Protein F1
Invasion	• Hyaluronic acid capsule • M protein
Spread through tissues	• Hyaluronidase • Streptokinase • SpeB (cysteine protease) • DNases A-D
Systemic toxicity	• Streptolysin O • Streptolysin S • Superantigen exotoxins

Particular attention has focussed on the M protein, a polypeptide chain complexed in an alpha-helical coil on the cell membrane. The M protein gene (emm) has been sequenced, and there are more than 124 recognised M genotypes now identified (emm types). Certain M serotypes have been associated with particular clinical syndromes.

Specifically, an M18 serotype was associated with an increase in rheumatic fever in the United States during the 1980's. Likewise, M1 and 3 have been frequently isolated in patients with severe invasive disease (Bisno et al., 2003).

The M protein has been the focus of extensive investigation in GAS pathogenesis. Consequently, it has been incriminated in the pathogenesis of rheumatic fever although the evidence for this is incomplete (discussed later).

Another important virulence factor related to resistance to phagocytosis is the ability of GAS to form a capsule, which is predominantly composed of hyaluronic acid (a high molecular weight polymer consisting of N-acetylglucosamine and glucuronic acid). Different streptococcal strains differ in their degree of encapsulation. The strains with better capsule production have a mucoid appearance on blood agar cultivation. The degree of encapsulation is associated with resistance to phagocytosis, and the ability to induce autoimmunity. Therefore, heavily encapsulated GAS strains (mucoid strains) are associated with acute rheumatic fever (Pilot I, 1944; Stollerman GH, 1996): In one study, only 3% of GAS strains causing uncomplicated pharyngitis had a mucoid morphology, whereas 42% of isolates from rheumatic fever were mucoid. The continuing incidence of rheumatic fever in Utah is partly attributed to the high rate of mucoid streptococci in the community (Veasy LG et al., 2004).

With relevance to autoimmunity, GAS capsular hyaluronate is a poor immunogen and antibodies against this product are rarely found in humans (Fillit HM et al., 1988). Encapsulation has also been proposed to promote more persistent throat colonisation (Wessels and Bronze, 1994).

A recent discovery is the recognition that GAS can internalise into epithelial cells (LaPenta D et al., 1994). The biological significance of internalisation is unknown,

although cellular penetration provides sanctuary from phagocyte, human antibody and certain antibiotics.

GAS strains also produce a range of extracellular products, particularly enzymes. Streptolysin O is a haemolysin, and is toxic to a variety of cells including leukocytes and heart tissue (Madden JC et al., 2001). DNases A-D are enzymes that liquefy pus and facilitate streptococcal spread. Hyaluronidase degrades hyaluronic acid in the host connective tissue. Antibodies to these extracellular products are an important part of the serodiagnosis of streptococcal infection.

GAS also produces pyrogenic exotoxins, which can act as bacterial superantigens. Superantigens are potent immune stimulators by causing excessive and non-specific T-cell activation. This results in excessive proinflammatory cytokine production, and an inflammatory cascade. This process is thought to be relevant to Streptococcal toxic shock syndrome (Bisno et al, 2003). The superantigen theory is not known to be relevant in rheumatic fever and related post-streptococcal neuropsychiatric disease.

1.3.2.Group A streptococcus in rheumatic fever

The most popular hypothesis regarding the pathogenesis of rheumatic fever (RhF) is that of molecular mimicry: the theory of antigenic similarity between constituents of GAS and human tissue (specifically heart, synovium and neuron), and subsequent cross-reactivity of the immune system between GAS and the host (discussed later). There are examples of such antigenic similarity in a variety of diseases, although there is incomplete evidence that these processes are pathogenically significant (Rose NR, 1998).

Different strains of GAS vary in their rheumatogenic potential, as best demonstrated by certain M serotypes being repeatedly associated with RhF (Bisno AL, 1980; Smoot JC et al., 2002). The rheumatogenic strains have distinct characteristics. Specifically,

they are commonly encapsulated and produce mucoid colonies. Also, the M protein molecules in rheumatogenic strains share particular antigenic domains, which are capable of mounting a strong IgG response in RhF patients (Bessen DE et al., 1990; Bessen DE et al., 1995).

1.4.The Basal Ganglia

1.4.1.Definitions and anatomy

The basal ganglia describe a group of nuclei located near the middle of the brain. The basal ganglia and other deep nuclei structures (including the thalami) are also referred to as the deep grey matter. The basal ganglia are further separated into the following regions:

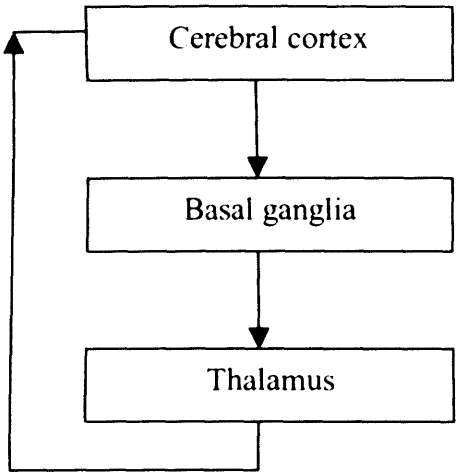
- Striatum (caudate and putamen)
- Pallidum (lateral and medial globus pallidus)

Other nuclei closely associated with the basal ganglia or sometimes included in the term 'basal ganglia' are the subthalamus and substantia nigra.

1.4.2.Functional organisation and basal ganglia circuitry

It has been demonstrated over the last 20 years that regions of the brain are best considered as parts of circuits, rather than discrete and unconnected areas (Graybiel AM, 1995). The basal ganglia form part of a circuit: the basal ganglia receive information from the cerebral cortex that subsequently outputs this information to the thalamus, which subsequently feeds back to the cerebral cortex (figure 1.1).

Figure 1.1. Simple schematic representation of basal ganglia circuitry.



However, it is apparent that this circuit is more complex and involves both direct and indirect pathways through the basal ganglia. Balance of these pathways is required for normal movements. If there is imbalance in these pathways, there could be resulting pathological over-excitation of the cerebral cortex (potentially resulting in hyperkinetic movement disorders) or over-inhibition of the cerebral cortex (potentially resulting in hypokinetic movement disorders). The primary neurotransmitters involved in this process are glutamate (excitatory) and gamma-aminobutyric acid (GABA- inhibitory) (Graybiel AM, 1995). A more accurate model of this circuitry is now presented. The direct (excitatory) and indirect (inhibitory) pathways are presented in figure 1.2 and 1.3 respectively. Figure 1.4 presents the combined pathways.

Figure 1.2. Direct pathway of basal ganglia circuitry. Activation of the direct pathway results in inhibition of the internal pallidum, consequent reduced inhibition of the thalamus, with consequent increased activation of the cerebral cortex (resulting in overall excitation).

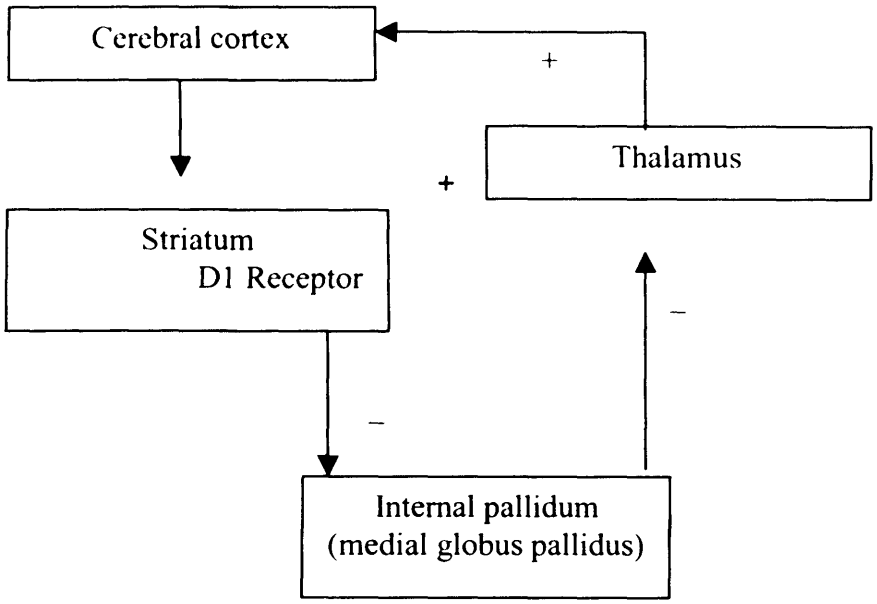


Figure 1.3. Indirect pathway of basal ganglia circuitry. Activation of the indirect pathway results in inhibition of the external pallidum (lateral GP), consequent reduced inhibition of the subthalamus, consequent increased excitation of internal pallidum, with consequent increased inhibition of the thalamus, with consequent reduced excitation of the cerebral cortex (inhibition).

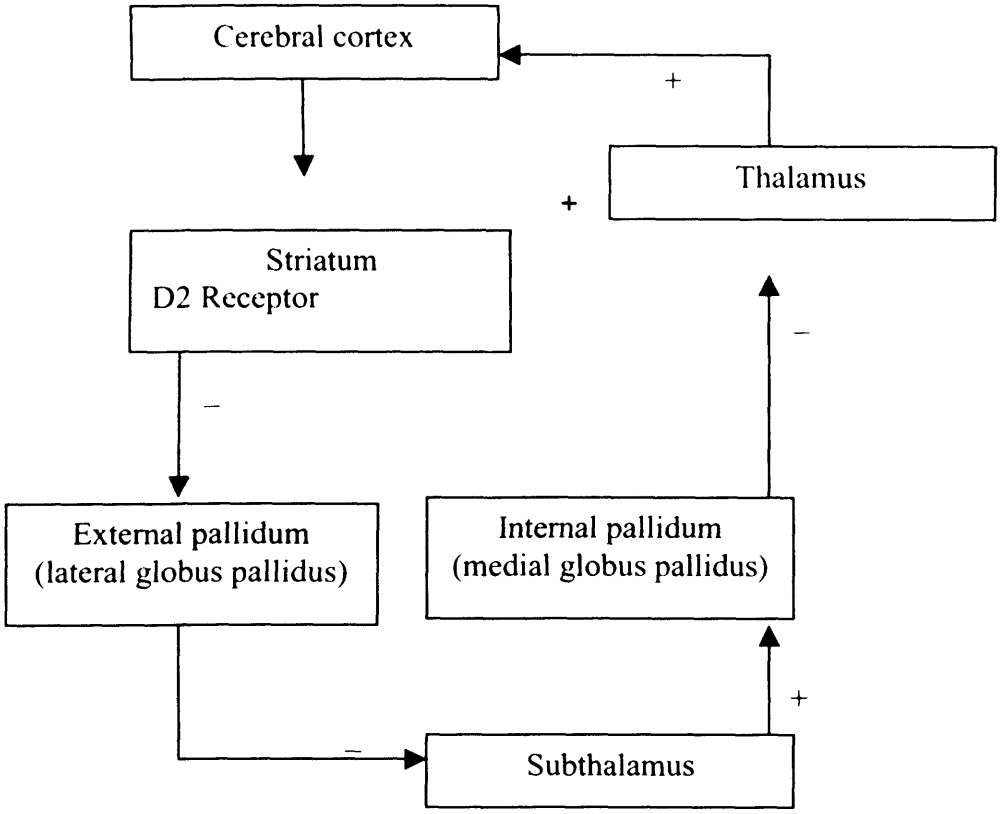
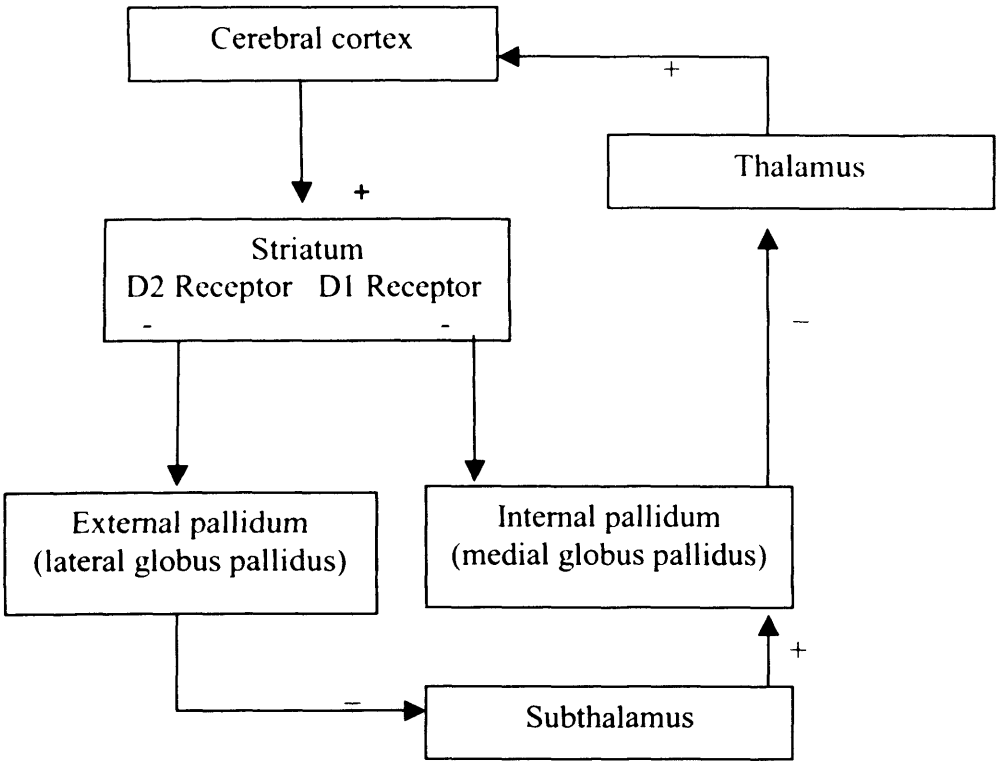


Figure 1.4. Direct and indirect pathways of basal ganglia circuitry, resulting (under non-pathological states) in a balance between excitation and inhibition (Graybiel AM, 2001).



1.5.Epidemiology and precipitants of Sydenham's chorea

1.5.1.Historical perspective and Thomas Sydenham

Thomas Sydenham (1624-1689) was a British physician referred to as the British Hippocrates. During the middle ages, a curious disease 'St. Vitus chorea' or 'chorea Sancti Viti' was described which was thought to be a hysterical response to religious superstition. Thomas Sydenham recognised a separate disorder that affected children. Confusingly, his original description in his last book Schedula monitoria de novae febris ingressa (Chapter XVI) was named 'On St. Vitus dance'. He recognised the disorder as separate to the hysterical dancing, however used the terminology 'chorea' and 'St Vitus dance' (Sydenham T, 1848). Subsequently, to aid differentiation and in respect to Sydenham, physicians preferred to use the term 'Sydenham's chorea'. Sydenham's original description was remarkably accurate. He described a childhood disorder ('from the tenth year to the time of puberty') presenting with a constellation of involuntary purposeless, rapid movements of the limbs, in addition to muscular weakness and emotional lability (Sydenham T, 1848). Sydenham initially thought chorea was due to 'some humour falling on the nerves' or 'such irritation causes the spasm' and suggested purging and bleeding of the victim to restore strength. However, Sydenham did not note the association with rheumatic fever (RhF). Indeed it was not until the 19th Century when the association with RhF was made. Etienne Michel Bouteille was the first to note an association between Sydenham's chorea and rheumatism in 1810. He noted that four of his patients had arthritic rheumatism before the onset of chorea, and one of his patients had chorea before rheumatism (Bouteille EM, 1810). In 1831, Sydenham's chorea was classified under the heading of rheumatism by Richard Bright (Bright R, 1831). After this, Germain See noted the

association, stating: 'chorea is the result of a rheumatic diathesis.' and 'for every two rheumatic children there is at least one who is choreic' (See G, 1850). The association of chorea with carditis and rheumatism was made by Henri Roger in 1866. He thought they shared a common source and suggested a condition of the blood or blood vessels was the cause, possibly secondary to thromboses and embolism (Straton CR, 1885; Greenfield JG and Wolfsohn JM, 1922). Straton was the first to associate chorea with infection and therefore a communicable disease in 1885 (Straton SC, 1885). A bacterial coccus was considered increasingly important in the late 19th century and Osler noted that 'Scarlet fever with arthritis may be a direct antecedent' (Osler W, 1894). In 1889, Westphal, Wassermann and Malkoff (Wesphal P et al., 1899) isolated a diplococcus from the pericardium and CSF of a child who died from chorea (with rheumatic pericarditis). Subsequently, others isolated an infectious coccus. Then in 1901-3, Poynton, Pane and Holmes produced an animal model of disease by injecting the diplococcus isolated from RhF patients into rabbits and producing a chorea-like disease, carditis and arthritis. They called it *Diplococcus rheumaticus* (Poynton F and Paine A, 1913).

By the mid 20th Century, chorea was thought to be part of an immuno-allergic response between the streptococcal toxin and host, with vulnerability of the synovial membrane, heart or nervous tissue.

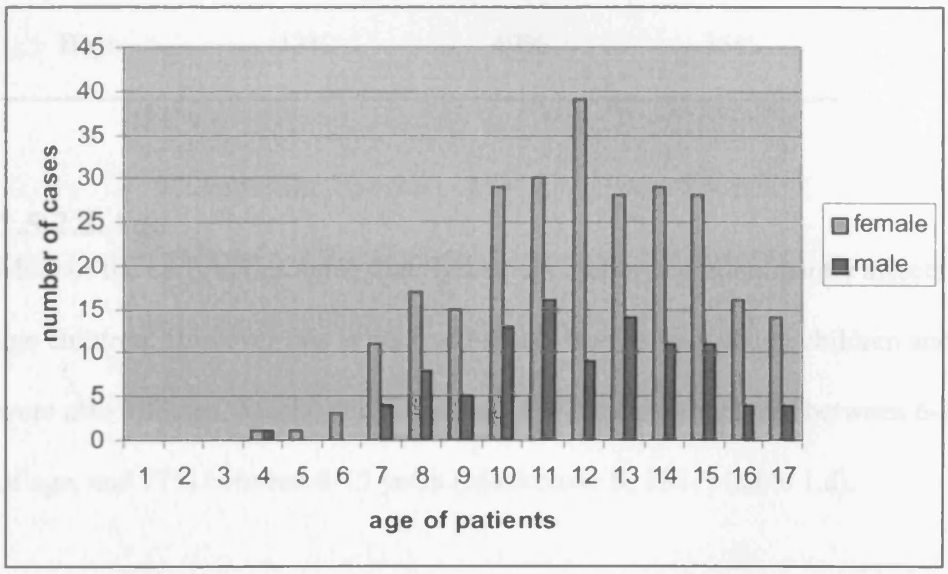
1.5.2.Epidemiology of Sydenham's chorea

Most of the epidemiological features of Sydenham's chorea can be gleaned from large case series published towards the end of the 19th century.

1.5.2.1.Sex

All of the literature agrees that females are more likely to develop Sydenham's chorea. Charles West reported his experience at Great Ormond Street Hospital, London in 300 females to 122 males (West C, 1865). Mackenzie similarly reported 322 females to 114 males (Mackenzie S, 1887) (Figure 1.5).

Figure 1.5. Age and sex distribution of Mackenzie's SC data (Mackenzie S, 1887).



This sex differential was manifest throughout all ages (Figure 1.5.). In US, Osler reported (in his Philadelphia series) 390 females to 161 males and reported that the predominance of females was much greater after puberty than in childhood (Osler W, 1894). West attributed the female vulnerability to a 'special excitability of the nervous tissue' in females. In the 20th century, Nausieda also reported a female predominance that was most manifest between the ages of 11-17 years (Table 1.c.) (Nausieda PA, 1980).

Table 1.c. Percentage of girls and boys with SC according to age (Nausieda PA, 1980).

Age	Total	3-10 years	11-17 years
Girls	57%	51%	65%
Boys	43%	49%	35%

1.5.2.2.Age

Most of the early series agree that Sydenham's chorea predominantly affects school age children. However this is not exclusively true as very young children and adults were also affected. Mackenzie found that 93% of cases occurred between 6-20 years of age, and 77% between 6-15 years (Mackenzie S, 1887) (table 1.d).

Table 1.d. Age of patients expressed as percentage of total (Mackenzie S, 1887).

Age (yrs)	0-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	>40
%	1.4	34	43	16	2	0.5	0.5	0.5	1.4

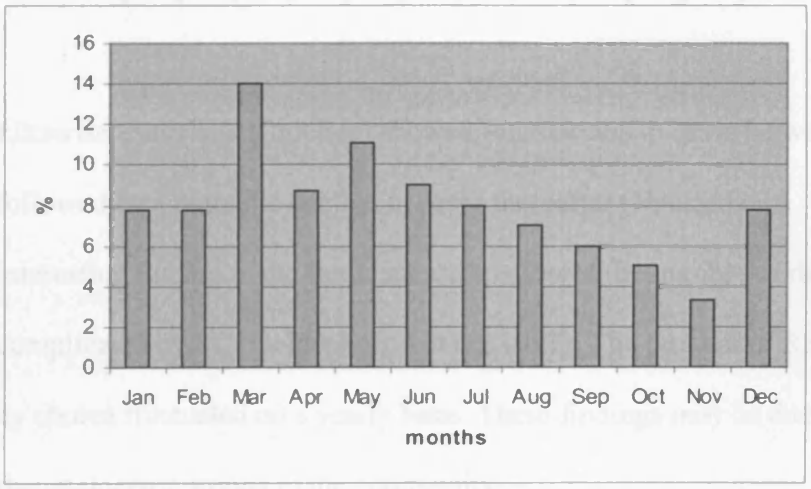
Very similar age distribution was described by West (GOSH), Osler (USA) and Nausieda (20th century cohort) with a peak around 9-10 years. However, Mackenzie described a number of cases presenting with first attacks aged in the 60's and 70's (Mackenzie S, 1887). The association of these cases with a carditis and the self-

limiting disease course makes the diagnosis of SC likely in these old-onset cases, rather than an undiagnosed hereditary or degenerative cause of chorea.

1.5.2.3.Seasonality

Before an association with streptococcal infection was made a number of observers had noted the association of SC with a seasonal distribution. A summary of Lewis's data reviewed by Osler is made in Figure 1.6 (Osler W, 1894). As can be seen more cases present during the colder months.

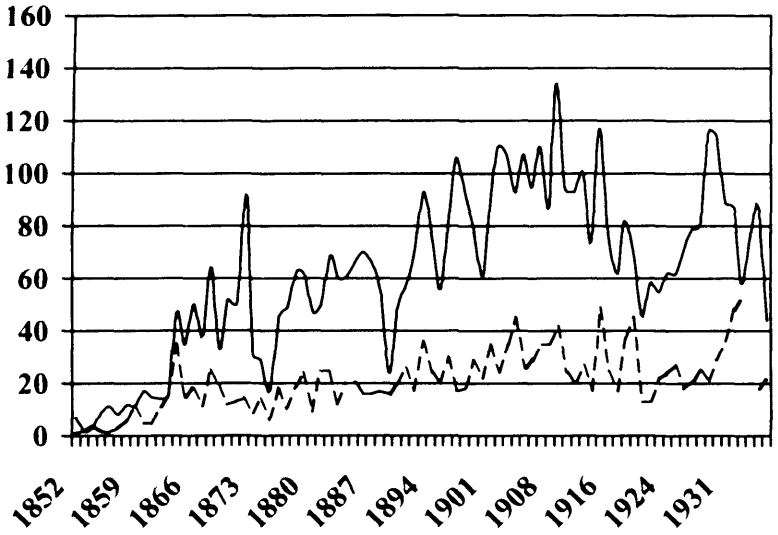
Figure 1.6. Seasonality of SC reported by Lewis (data from Jun-Oct are extrapolated from text). Percentage of cases plotted against months of year (Osler W, 1894).



1.5.2.4.Year fluctuations

It has been noted for over a century that the cases of SC fluctuate every few years. A review of the Great Ormond Street hospital cases by Davide Martino demonstrated this fluctuation that approximately had a 3-4 yearly pattern (Figure 1.7) (Martino D, with permission). As seen in Figure 1.7 not only does the number of RF patients fluctuate on a yearly basis, but the proportion of RF patients that get SC also fluctuates yearly.

Figure 1.7. Early fluctuations in SC cases seen at Great Ormond Street Hospital between 1852 and 1931 (Martino D, with permission). SC cases unbroken line, rheumatic fever cases broken line.



Likewise, Nausieda (Chicago) showed fluctuations in cases between 1951-1968, followed by a dramatic decline in cases thereafter (Nausieda PA, 1980). Another interesting finding is the fluctuant association of the number of rheumatic fever cases complicated by SC (Goldenberg J et al., 1992). The number of RF cases complicated by chorea fluctuated on a yearly basis. These findings may be due to changes in the rheumatogenic strains in the community.

1.5.2.5. Socio-economic class and race.

Reports from the 19th Century noted that, although children from all grades of society were susceptible to SC, children from lower classes were most likely to get the disease. Mackenzie reported that 70.5% of his cases were from the lower classes, data supported by Osler (USA) (although no epidemiological data was available for

comparison) (Mackenzie S, 1887; Osler W, 1894). In the 20th Century, the vulnerability of the lower classes was attributed to overcrowded homes (Coburn AF, 1931). Studies of army recruits showed that the incidence of positive streptococcal pharyngitis was inversely proportional to the distance between beds in army barracks (Wannamker LW, 1953), and subsequent antibiotic use in these populations reduced the incidence of rheumatic fever (Frank PF et al., 1965).

Although initial reports by Osler in the 20th Century proposed that black and Indian races were less likely to develop SC, the clear endemic status of SC and rheumatic fever around the World (South America, Africa, Asia, Aborigine populations of Australia) makes this statement less tenable. It appears all races are vulnerable to SC, and other criteria (overcrowding, antibiotic use, genetic vulnerability) are more important risk factors.

1.5.3.Precipitants

Early reports throughout the 19th Century described a psychological fright was the most likely precipitant of chorea. Indeed 'fright chorea' was considered the cause in ~15% of cases such as 'over work at school' or 'events at home' (Osler W, 1894; West C, 1865). Also when multiple cases occurred on hospital wards at the same time, these cases were often attributed to hysteria (Osler W, 1894). Other proposed precipitants included trauma and blows to the head, and even eye-strain secondary to hypermetropism or myopia (Osler W, 1894). However, by the 1880's reports were noting the association with 'erosions of the mucous membranes of the nose and throat (Straton CR, 1885). Straton postulated that these erosions could afford portals of entry for micro-organisms which might be the indirect cause of chorea. Thereafter the

association with tonsillitis became more apparent. In 1889, Cheadle reported that tonsillitis 'ushered in' acute rheumatism in ~24% of cases, and was often very closely associated with the onset of arthritis (Cheadle WB, 1889). Likewise, Scarlet fever was an antecedent illness in 129 of 432 cases by Mackenzie (Mackenzie S, 1887). Cheadle also noted the association of chorea with scarlet fever, and stated by contrast 'we never see a measles chorea, or whooping cough chorea, or mumps chorea (Cheadle WB, 1889). Cheadle's report also highlighted the clear association of preceding rheumatic arthritis. Shortly after these reports, Group A streptococcus (GAS) was directly linked to SC and rheumatic fever pathogenesis. As the association of rheumatic fever/SC with GAS infection became manifest in the 20th Century, few investigators have subsequently reported the antecedent illnesses in SC.

1.6.Clinical spectrum of post-streptococcal neuropsychiatric syndromes

1.6.1.Movement disorders- Sydenham's chorea

The best described of all movement disorders after GAS infection remains chorea. Chorea is Greek for dance, and Sydenham described a constellation of involuntary, purposeless rapid movements of the limbs. The early characteristics of SC described by West included the child being 'awkward and fidgety', followed by stumbling whilst walking and involvement of the face with the 'strangest grimaces' (West C, 1865). Like most extrapyramidal movement disorders, the movements are exacerbated by excitement, and cease during sleep. Extra movements of the hands associated with hypotonia results in the 'milkmaid's grip' (inability to sustain the grip resulting in intermittent squeezing contractions). Involvement of the tongue results in hurried and unsustained tongue movements, with frequently affected oropharyngeal

involvement and consequent stammering, impaired swallowing and dysarthria (West C, 1865).

The chorea is usually generalised: Mackenzie reported that in only 30 of 429 cases were the movements entirely one sided or predominantly one sided (hemichorea) (Mackenzie S, 1887). Other more recent reports describe hemichorea in 19% (Nausieda PA, 1980). Other features include hypometric saccades, and occasionally oculogyric crises (Cardoso F et al., 1997). Other co-morbid movement disorders in SC in addition to chorea have been described. Indeed myoclonus and tics have been described since the 19th Century (Creak M and Guttman E, 1935; Osler W, 1894). Recently tics have been described in SC, sometimes rarely (Cardoso F et al., 1997) and sometimes very frequently (Mercadante MT et al., 2000). Mercadante described motor tics in 73% of a Brazilian cohort of SC (n=22). This has particular relevance to the later discussion on the PANDAS hypothesis (Mercadante MT et al., 2000).

1.6.2.Other movement disorders other than chorea- the PANDAS hypothesis

Basal ganglia disorders rarely conform to one extrapyramidal phenotype alone. For example, in Huntington's disease, tics, dystonia and Parkinsonism may occur, although chorea remains the most characteristic phenomenon (Jankovic J et al., 1995; van Dijk JG et al., 1985). As mentioned in the previous section, myoclonus has been described in SC, and differentiating myoclonus from chorea may sometimes be difficult. A recent report with videotape described a case of myoclonus after streptococcal infection (DiFazio MP et al., 1998). However, most recent interest has focussed on the description of motor tics after streptococcal infections. A number of groups had previously described isolated cases of tics and Tourette's syndrome after

streptococcal infections (Creak M and Guttman E, 1935, Kerbeshian J et al., 1990; Kiessling LS et al., 1993). Then in the 1980's, an outbreak of Group A streptococcal tonsillitis in Rhode Island was associated with a 10-fold increase in the incidence of motor tics (without chorea): the concept of post-streptococcal tics was born (Kiessling LS et al., 1993). A separate group of children were followed in the late 1980's and 1990's with neuropsychiatric disorders (defined by tics and obsessive-compulsive disorder) who had exacerbations following streptococcal infections. They had a relatively unique course because of the abrupt onset of symptoms or dramatic exacerbations that 'came on overnight' (Allen AJ et al., 1995; Swedo SE et al., 1998; Leonard HL and Swedo SE, 2001). The important and defining nature of these patients was the temporal association between exacerbations and further streptococcal infections. It was thought that the children would exhibit tics or OCD if the 'dose' of the aetiological agent were insufficient to cause frank chorea (Swedo SE, 1994). Sue Swedo from National Institute of Mental Health (USA) coined the term PANDAS (Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections). This group established working criteria for a diagnosis of PANDAS as follows (Swedo SE et al., 98):

1. Presence of OCD and/ or tics (DSM-IV)
2. Paediatric onset (pre-pubertal)
3. Episodic course with abrupt onset and dramatic exacerbations (frequently explosive)
4. Association with GAS infection (either throat culture or elevated anti-GAS antibodies). The authors noted that although there may be significant lag between GAS infection and the onset of the 1st episode, the lag of days between infection and subsequent relapses or exacerbations is much shorter,

often just days. Swedo noted that when GAS serology is used, not only must seropositivity be associated with symptom exacerbations, but also that seronegativity (or falling titres) is associated with symptom remission (Swedo SE et al., 1998).

5. Association with neurological abnormalities. They defined these as adventitious movements or motoric hyperactivity. They also used the term 'choreiform movements' which is a confusing terminology but basically means chorea-like but not frank chorea.

An important criteria was the absence of systemic features (carditis, arthritis). In 1998, Swedo et al. published their findings of 50 children conforming to the PANDAS criteria (Swedo SE et al., 1998). Of these, 80% had motor tics and there was a male:female predominance of 2.6:1. Both the tics and choreiform movements waxed and waned in severity over time, and exacerbations were temporally linked to streptococcal infections. The children had significant associated co-morbid psychiatric disturbance that will be described in detail later. The defining GAS infection was present at symptom onset in 42% of the 50 children. Every child had at least one symptom exacerbation that was preceded (within 6 weeks) by a documented GAS infection. In total there were 144 exacerbations in 50 children where the association with GAS infection was known. 23% of these exacerbations were not associated with any sign of GAS infection in the previous month. The possibility that viral or other infections may be causing exacerbations was proposed (Swedo SE et al., 1998).

Clearly there are a number of weaknesses with the current PANDAS criteria including (Kurlan R, 2004):

- GAS infection is extremely common in paediatric populations and may be incidental (particularly in winter) (Kaplan EL and Gerber MA, 1998)
- Tic disorders tend to wax and wane naturally. Sudden deteriorations are also possible: 53% of unselected tic patients had a history of sudden or explosive onset or worsening of tic symptoms (Goetz CG et al., 1992).
- Most tics have onset pre-pubertally. Therefore this is unlikely to be a discriminating marker of PANDAS.
- The terminology of choreiform movements is ambiguous and some commentators assumed that these cases were just Sydenham's chorea (Kurlan R, 2004). Swedo defends this by defining choreiform movements as fine piano playing movements that are absent at rest and only elicited in stressed postures, whereas choreatic movements are present continuously and associated with failure to sustain tetanic contractures (milk maid's grip) and muscle weakness (Swedo SE et al., 2004).
- Why do PANDAS patients not have systemic involvement (arthritis, carditis) if the immune mechanism is similar to SC/rheumatic fever? This important question has not been adequately examined.
- The latency between GAS infection and SC onset is often 3-5 months. A similar latency in PANDAS would make a temporal association between GAS infection and neuropsychiatric disease onset difficult.
- Swedo herself warned of the pitfalls of using a single anti-streptococcal serodiagnosis at the time of tic exacerbations (Swedo SE et al., 1998). Instead longitudinal laboratory data is required with rising titres associated with symptom exacerbation, and falling titres on symptom remission.

Likewise culture of GAS may occur in asymptomatic carriers and could lead to misdiagnosis.

Despite these criticisms, the concept of PANDAS has generated broad interest amongst physicians and lay people alike.

1.6.3.Psychiatric disorders

1.6.3.1.Sydenhams' chorea

The high incidence of emotional factors in Sydenham's chorea has been recognised for over a century, and was initially referred to as a 'choreic temperament'. However, further evaluation of the psychiatric sequelae of Sydenham's chorea demonstrated that diagnosable emotional and disruptive disorders were common accompaniments. A follow-up study showed high prevalence of psychiatric syndromes in 75% of patients (Freeman JM et al., 1965). Specifically, the study followed up patients with unequivocal SC (n=40) after a mean of 29 years (22-34 years) and compared the follow-up psychiatric morbidity with a control group of patients from the same era with glomerulonephritis, osteomyelitis and rheumatic fever (n=30). A summary of the findings is presented in table 1.e. As can be seen, there was a significantly higher rate of psychiatric problems in the SC patients compared to controls.

Table 1.e. Psychiatric disorders in SC and controls (Freeman). Definitions by Freeman (Freeman JM et al., 1965).

Psychiatric disturbance	SC (n=40)	Controls (n=30)
No psychiatric disturbance	7/40 (17 %)	22/30 (73%)
Personality disorder (passive dependency, passive aggressive, compulsive, inadequate personality, sexual deviant, others with marked depressive, anxiety or hysterical features)	26/40 (65%)	8/30 (27%)
Definite psychoneurosis (phobia, obsessive-compulsive disorder, conversion, anxiety)	7/40 (17%)	0/30 (0%)

More importantly, this study demonstrated that the behavioural consequences often remained for decades after the resolution of childhood chorea (Freeman JM et al., 1965). More recent examination of SC cohorts revealed a high incidence of obsessive-compulsive behaviours (Swedo SE et al., 1989; Asbahr FR et al., 1998). Swedo compared obsessive-compulsive symptoms (OCS -using the Leyton Inventory) in SC patients (n=23) compared to rheumatic fever without chorea (n=14). The SC patients had significantly higher obsessionality scoring than the rheumatic fever patients and higher interference scores (impairment). Three of the SC patients (13%) had clinically diagnosable obsessive-compulsive disorder. Notably, the SC patients also had significantly higher depression and anxiety scores than the rheumatic fever patients (Swedo SE et al., 1989). A separate study by Swedo (Swedo SE et al., 1993) examined the psychological features of 11 children with acute SC. She described an

abrupt onset of severe nightmares, decreased attention span and emotional lability (described as 'moody, clingy, crying and tantrums'). During their illness, nine of 11(82%) met diagnostic criteria for OCD, although most symptoms resolved at follow-up after resolution of the chorea. Although not all studies have documented a high rate of OCS in acute SC (Faustino PC et al., 2003), a subsequent study further established a link between SC and obsessive-compulsive symptoms (OCS) (Asbahr FR et al., 1998). This study examined the prevalence of OCS during the acute phase of SC (n=30) compared with rheumatic fever without chorea (n=20). 70% of the SC patients had OCS peaking during the 2nd month of whom 16.7% met criteria for OCD at that time (Asbahr FR et al., 1998). The OCS presented before the chorea in 28.6%, although waned and disappeared in the majority. By contrast, 0% of the rheumatic fever patients without chorea had OCS at any time. Furthermore, the incidence of OCD appeared to be increasingly common in children who had relapses of SC (Asbahr FR et al., 1999). Subsequent analysis of cohorts of SC has demonstrated that psychiatric comorbidity is not limited to OCD, but includes other emotional disorders such as generalised anxiety, separation anxiety and major depression (Mercadante MT et al., 2000). The prevalence of psychiatric diseases reported by Mercadante is presented in table 1.f.

Table 1.f. Psychiatric disorders in SC and controls reported by Mercadante (Mercadante MT et al., 2000).

Psychiatric disorder	Control group (various medical problems) (n=20)	Rheumatic fever (n=20)	SC (n=22)
OCD	0%	10%	14%
OCS	0%	25%	32%
ADHD (combined)	0%	0%	46% *
Major depression	0%	0%	41% *
Separation anxiety	10%	5%	14%
Generalised anxiety	0%	5%	9%

* SIGNIFICANT DIFFERENCE

OCD: obsessive-compulsive disorder
OCS: obsessive-compulsive symptoms
ADHD: attention deficit hyperactivity disorder

In addition, this group showed that the presence or absence of preceding ADHD symptoms was a predictor for the risk of developing SC. If a child had an antecedent history of ADHD (combined type) they had a significantly higher risk of developing SC (85%) than the rheumatic fever controls (36%) (Mercadante MT et al., 2000). Swedo (Swedo SE et al., 1993) also described a broader psychiatric phenotype in SC with separation anxiety, oppositional disorders and ADHD all over-expressed in her 11 SC patients. Likewise Faustino found attentional problems and aggressive behaviour in their SC patients (n=19) (Faustino PC et al., 2003).

There had been a number of reports of schizophrenia as an outcome of SC (Wertheimer N. 1961), although recent literature has not repeated this observation (Swedo SE et al, 1989, Swedo SE et al., 1993, Swedo SE et al., 1998).

1.6.3.2.PANDAS

The psychiatric morbidity of PANDAS is similar to (if not identical to) Sydenham's chorea. In the acute stages, there is typically a rapid alteration in behaviours and emotional lability (Swedo et al., 1998). Indeed Swedo pointed out the explosive onset and dramatic deteriorations should be required for diagnosis. PANDAS was defined as post-streptococcal emergence of OCD and/or tics. Indeed OCD and/or tics were required for a diagnosis of PANDAS, therefore it is not possible to directly compare the incidence of OCD in PANDAS cohorts with SC cohorts. There was a broad range of obsessions (contamination, harm, hoarding, somatic, religious) and compulsions (washing, checking, repeating, counting, ordering, hoarding) as seen in idiopathic OCD. However analysis of 50 patients with PANDAS demonstrated a high incidence of other emotional disorders (major depression 36%, separation anxiety 20%), conduct disorders (oppositional defiant disorder 40%) and attention deficit hyperactivity disorder (40%) (Swedo et al., 1998). By contrast mania, psychosis and somatization disorders were not present. In summary, a broad range of emotional, attentional and conduct disorders are seen in both SC and PANDAS, although the classic psychiatric syndrome of both remains OCD.

1.6.4. Other CNS features

1.6.4.1. Sydenham's chorea

The clinical outcomes would appear to be relatively specific to extrapyramidal movement and psychiatric disorders. Dysarthria and hypotonia are common accompaniments, and would be considered characteristic of SC. Indeed dysarthria occurs in 15-100% of series and is considered to be extrapyramidal in origin (Nausieda PA et al., 1980, Swedo SE et al., 1993, Cardoso F et al., 1997). The

hypotonia is often mild, although occasionally it can be profound with apparent paralysis, sometimes known as chorea paralytic or chorea mollis (Marques-Dias MJ et al., 1997). A more disseminated encephalomyelitis with confusion, disorientation and rarely delerium is reported in 10% of cases (Nausieda PA et al., 1980). Occasional reports of acute disseminated encephalomyelitis have been reported after streptococcal infection (Jorens PG et al., 2000). Seizures are rarely reported 0.4-0.5% (Nausieda PA et al., 1980; Mackenzie S, 1887), likewise cranial nerve palsy in 0.4% only (Mackenzie S, 1887). Cerebellar or sensory abnormalities, mutism, dementia and visual impairments are rarely or never seen (Cardoso F et al., 1997).
Neuropsychology has not revealed any significant cognitive disturbance although one study highlighted some equivocal impairment of frontal lobe functioning (Swedo SE et al., 1993).

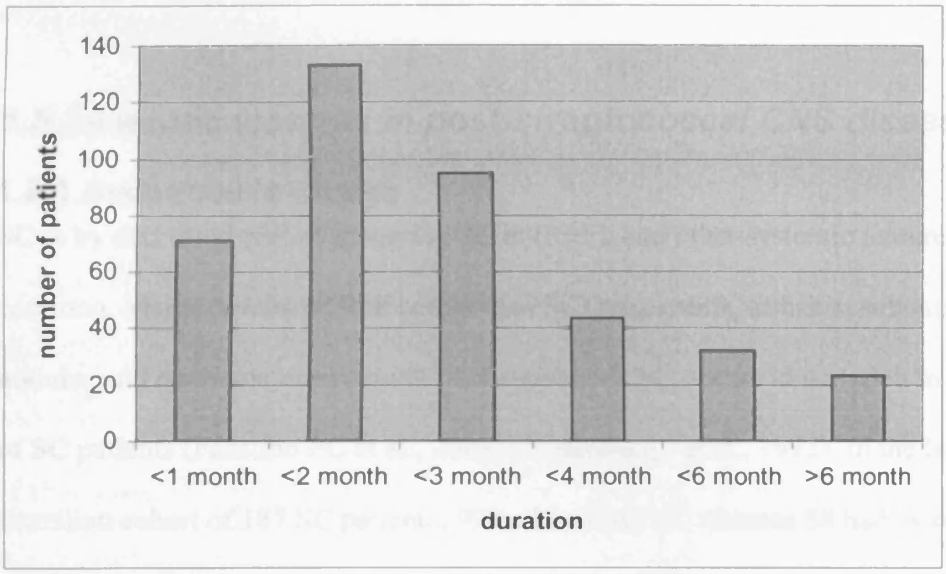
1.6.4.1. PANDAS

In PANDAS, other features described by Swedo included enuresis (12%), encoporesis (10%) and deterioration in math skills (26%) (Swedo SE et al., 1998).

1.7. Clinical outcome

The standard medical texts describe that SC is a benign monophasic disorder that self-terminates in the majority. Review of the literature finds that this statement is mainly true. Mackenzie found that the usual duration of attacks is 2-3 months, a finding confirmed by Nausieda in 1980 (Figure 1.8) (Mackenzie S, 1887; Nausieda PA et al., 1980). However a recent study of SC from Brazil found that SC persisted longer than 2 years in half of patients (n=32) (Cardoso F et al., 1999). In this Brazilian report, some patients were not aware of the chorea (Cardoso F et al., 1999).

Figure 1.8. Nausieda data on the duration of chorea (Nausieda PA et al., 1980).



When present, relapses of SC are usually isolated and occur most commonly in the first year after onset, or during pregnancy (chorea gravidarum) (Mackenzie S, 1887). The fact that pregnancy and the oral contraceptive pill can precipitate relapses suggests a role of oestrogen in SC expression (Cardoso F, 2002). It should be noted that persistent psychiatric sequealae are common, even after the apparent resolution of chorea as previously discussed (Freeman JM et al., 1965).

SC is rarely fatal, in one large report (n=439) from the late 19th Century, 2% of cases were fatal, almost all attributable to co-existing heart disease (Mackenzie S, 1887).

By definition, PANDAS has a relapsing remitting course with temporal association after further infections (Swedo SE et al., 1998). Although streptococcal infections appear to be the initiators of disease, other infective triggers may be responsible for relapses (Allen AJ et al., 1995; Berrios X et al., 1985).

Although there are few longitudinal studies examining the natural history of disease, it is likely that the outcome of both SC and PANDAS is variable, varying from

monophasic self-limiting disease, to a persistent relapsing remitting course over many decades.

1.8.Systemic features in post-streptococcal CNS disease

1.8.1.Sydenham's chorea

SC is by definition part of rheumatic fever (RhF), and other systemic features are common. Major criteria of RhF (other than SC) are carditis, arthritis, subcutaneous nodules and erythema marginatum (Jones criteria). SC occurs in isolation in 53- 74% of SC patients (Faustino PC et al., 2003; Goldenberg J et al., 1992). In the large Brazilian cohort of 187 SC patients, 99 had isolated SC whereas 88 had associated symptoms (carditis and arthritis in n=32, arthritis alone in n=30, carditis alone n=26) (Goldenberg J et al., 1992). Notably this group demonstrated an elevated ASOT more commonly in the SC patients with systemic features, than those with isolated SC (83 v. 43% $p<0.01$). Also a positive throat culture for Group A streptococcus was uncommon in the isolated SC group (11%) suggesting that the isolated SC group had a longer latency after infection, by which time the host had frequently cleared the organism.

1.8.2.PANDAS

By definition, PANDAS patients should not have any evidence of rheumatic fever (Swedo SE et al., 1998). However this definition was produced to improve diagnostic differentiation of PANDAS from SC, rather than a proven pathological distinction. One study to address this issue has been published only in abstract form: a group performed echocardiography in PANDAS patients (n=36). They found a high incidence of asymptomatic mild or minimal mitral regurgitation (the commonest cardiac lesion seen in rheumatic fever) in 47% of PANDAS patients, persisting at 1

year (Cardona F et al., 2003). This suggests that asymptomatic systemic disease may occur in PANDAS, and indicates that PANDAS may be associated with rheumatic fever.

1.9.Genetic predisposition

1.9.1.Sydenham's chorea

A high incidence of 'nervous affections' has been reported in families of patients with SC for over a century. Indeed Mackenzie reported that 46% of SC family members had a nervous affection of some kind (Mackenzie S, 1887) (particularly the parents or siblings). However, the definition of nervous affections was very broad and encompassing, including idiocy, epilepsy, drunkenness and paralysis. Notably nervousness and excitability occurred most commonly in family members (Mackenzie S, 1887). Osler reported chorea in the family members of 80 of 554 families (14%) (Osler W, 1894). Likewise, a family history of SC occurred in 13% in Nausieda's series (n=162), and 26-36% had a family history of rheumatic fever (Aron AM et al., 1965; Nausieda PA et al., 1980).

1.9.2.PANDAS

In PANDAS, the incidence of rheumatic fever has not been evaluated, and therefore comparisons with the SC data cannot be made. However, one study examined the incidence of movement and psychiatric disorders in parents and siblings of PANDAS patients: 39% had at least one first-degree relative with a history of motor or vocal tics, and 26% had at least one first degree relative with OCD (Lougee L et al., 2000). In summary, there is a positive family history of psychiatric, movement and rheumatic fever in a significant proportion of patients with SC and PANDAS. This supports the

hypothesis that post-streptococcal CNS disorders are multifactorial involving a genetic predisposition plus exposure to streptococcal infections.

1.10. Other 'post-infectious' movement disorders

1.10.1.Encephalitis lethargica

'Lethargic encephalitis' has been described for many centuries by physicians such as Hippocrates and Sydenham. The most recent epidemic ravaged the World between 1916-1927, and was named 'Encephalitis lethargica'-EL by the clinician most associated with this disorder- von Economo (von Economo C, 1931). The classic phenotype in EL was Parkinsonism, although dystonia, tics and chorea were also common and co-existed. The disorder was often preceded by pharyngitis. In addition to the extrapyramidal movement disorders, von Economo noted a sleep disorder, lethargy and neuropsychiatric disorders were common in the survivors (e.g. catatonia, obsessive-compulsive disorder, depression and mutism). Additional common features included oculogyric crises, ocular features (ophthalmoplegia and ptosis) and central cardiorespiratory features (particularly hiccough). Von Economo felt that the clinical phenomenology localised the disease to the basal ganglia and brainstem. Pathological findings, like the clinical characteristics, demonstrated an encephalitis with particular involvement of the basal ganglia and midbrain structures.

Because EL was epidemic during the same time period as the 1918 influenza pandemic, an association between the two diseases has been proposed (Ravenholt and Foege, 1982). However, von Economo and other workers during the EL epidemic thought that influenza was not the cause. In addition, patients with EL rarely had influenza before neurological onset; in one early report of 76 EL cases, only 4 had influenza in the 6 months preceding onset (Stallybrass CO, 1923).

Recent discovery and identification of the 1918 influenza virus genome by Taubenberger (Taubenberger JK *et al.*,1997) allowed this group the opportunity to examine archived EL brain specimens for 1918 influenza virus RNA. They failed to find influenza RNA in EL brains, and furthermore determined that the 1918 influenza virus was genetically incapable of neurotropic disease and only capable of reproduction in the respiratory tree. The authors concluded that EL was unlikely to have been directly due the 1918 influenza virus (McCall *et al.*, 2001). A further group also failed to demonstrate the 1918 influenza virus in archived EL brains (Lo KC et al., 2003).

There have been no further epidemics of EL since the 1920's although sporadic cases have continued to be reported (Rail *et al.*, 1981; Howard and Lees, 1987; Blunt *et al.*, 1997; Kiley and Esiri, 2001). Contemporary reports have consistently failed to demonstrate evidence of neurotropic viral particles, and the cause of EL remains unknown. However, a common finding on CSF examination includes the presence of IgG oligoclonal bands (Williams *et al.*,1979; Howard and Lees, 1987; Kiley and Esiri, 2001). In addition, some recent cases have been successfully treated with steroids (Blunt *et al.*, 1997). These features have lead investigators to propose that this phenotype may be a post-infectious immune-mediated CNS disorder. Interestingly, during the epidemic attention had focused on streptococcus as a possible etiological agent. A number of investigators had detected streptococcus in the throats of patients suffering from EL (Wilson, 1918; Harris, 1918; von Economo, 1931). In addition, they were able to induce an encephalitis lethargica-like illness in dogs after vaccination with streptococcal organisms (von Economo, 1931). However, as gram positive organisms were never isolated from the brain, the authors concluded that it

was unlikely to have been the primary etiological agent. The description of an EL phenotype after streptococcal infection is reported in this thesis.

1.10.2.Tourette syndrome and obsessive-compulsive disorder

Tic disorders, Tourette's syndrome and obsessive-compulsive disorder are considered to be variable phenotypic expressions of the same brain disorder. The pathophysiology is thought to be secondary to dysfunction of cortico-striatal circuits, although the exact pathology is unknown (Stein DJ, 2003). It is likely that a variety of processes can disrupt this circuit and result in these clinical phenotypes. The recognition of PANDAS has lead to speculation that subgroups of 'idiopathic' tic disorders, Tourette's syndrome (TS) and OCD have a (post-streptococcal) immune mediated pathophysiology. Using streptococcal serology, a number of researchers have attempted to investigate a possible association of streptococcal infection with tic disorders and TS. The studies have generally measured streptococcal serology in cohorts and compared them to control groups. The studies are reviewed in table 1.h.

Table 1.h. Studies investigating streptococcal serology in Tourette's syndrome (TS) cohorts compared to controls.

Reference	Cohort (number of patients)	Control group (number of controls)	Comment
Singer HS et al., 1998 (US) *	Tourette (n=41)	Hospital and community tic-free (n=39)	No difference in ASOT and anti-DNAse B titres.
Kiessling LS et al., 1993 (US)	Movement disorder (MD), tics, TS, choreiform (n=24)	ADHD, learning difficulties but no movement disorder (n=26)	MD cohort significantly elevated ASOT and antiDNAse B.
Muller N et al., 2000 (Germany)	Child TS (n=13) Adult TS (n=23)	Healthy children (n=12) Healthy adult (n=24) Adult schizophrenia (n=17)	ASOT and anti-DNAse B elevated in TS.
Muller N et al., 2001 (Germany)	Adult TS (n=25)	Adult healthy (n=25)	M12 and M19 streptococcal antibodies elevated in TS.
Cardona F et al., 2001 (Italy)	Childhood tics (n=150)	Phenylketonuria and epilepsy, no tics (n=150)	Elevated ASOT in tic cohort.
Church AJ et al., 2003 (UK)	Childhood TS (n=56) Adult TS (n=44)	Child streptococcal infection (n=40) Child neurology (n=50) Adult healthy (n=50) Adult neurology (n=50)	Significantly elevated ASOT in child and adult TS cohorts.
Loiselle CR et al., 2003 (US) *	Child TS (n=41)	Healthy children (n=38)	No difference in whole cohort. Significantly higher ASOT in TS with comorbid ADHD.
Morshed SA et al., 2001 (US)	TS (n=81)	SC (n=27) Autoimmune disorders (n=52) Normal (n=67)	ASOT significantly higher in TS and SC compared to controls.
Singer HS et al., 1999 (US) *	TS (n=41)	Controls no tics (n=39)	No difference in ASOT and anti-DNAse B titres.

*These cohorts were all Singer (US) and may be the same samples from the same cohorts.

As can be seen, a number of the studies demonstrated elevated streptococcal serology in tic and TS cohorts compared to controls, although not all of the cohorts demonstrated this association. In fact, the negative studies tended to be from Singer's group in the US, unlike studies from Germany, Italy, Britain and other US groups. Indeed, the studies from Singer 1998 and 1999 appear to have used the same cohorts in both studies (and possibly also Loiselle 2003). One study demonstrated a positive correlation between ASOT and the severity of tics (Cardona F et al., 2001). At present, it is difficult to know the exact proportion of TS who may have an association with streptococcal infections. Furthermore, due to the natural tendency for TS to wax and wane, longitudinal studies are required.

1.11.Neuroimaging in post-streptococcal CNS syndromes

Neuroimaging often provides insight into the localisation of CNS disease, although it rarely provides detailed biological insight into the pathogenesis. Imaging of the brain using conventional CT and MRI is commonly normal in post-streptococcal CNS disease (Giedd JN et al., 1995; Swedo SE et al., 1993). In Giedd's SC study only 2/24 had an abnormality on conventional MR imaging (Giedd JN et al., 1995), and 5/11 in Swedo's SC study had any abnormality (although most of these were likely to be incidental and of no clinical significance) (Swedo SE et al., 1993). One study reported a higher incidence of MRI abnormalities (42%) in 19 SC patients, although the majority of these were probably incidental non-specific abnormalities such as small white matter signal changes or arachnoid cysts (Faustino PC et al., 2003). Occasionally, inflammatory changes have been described in SC, which are predominantly (but not exclusively) localised to the basal ganglia (Kienzle GD et al., 1991; Castillo M et al., 1999; Traill Z et al., 1995; Robertson W et al., 2002). Only

rarely has enhancement after contrast been described, suggesting that disruption of the blood brain barrier is not typical (Kienzle GD et al., 1991). More sophisticated techniques including volumetric studies have shown that the caudate and putamen are specifically enlarged during the acute phase of Sydenham's chorea and PANDAS (Peterson BS et al., 2000; Giedd JN et al., 1995; Giedd JN et al., 2000). By contrast, there were no differences in the volume of the total hemispheres, prefrontal or midfrontal regions, or thalamus. Furthermore, anecdotal evidence has shown that basal ganglia size correlates with the disease course; a longitudinal case study demonstrated striatal enlargement during acute presentation with normalisation during remission (Giedd JN et al., 1996). SPECT studies have shown hypermetabolism and increased glucose consumption in the basal ganglia during acute SC (Goldman S et al., 1993; Weindl A et al., 1993; Lee PH et al., 1999). Furthermore, a case report of SC using MR proton spectroscopy has shown reduction of n-acetyl aspartate in the striatum, which was interpreted as indicative of neuronal dysfunction or loss (Castillo M et al., 1999). Although the majority of imaging findings are reversible, occasionally irreversible striatal changes have been described suggesting permanent damage is possible (Emery ES et al., 1997; Ikuta N et al., 1998). Although the majority of imaging studies have localised disease to the basal ganglia, imaging is rarely useful as a diagnostic tool alone, and is more commonly used to exclude other causes of movement disorders. The imaging studies also suggest that there is a spectrum of immune mediated brain pathology, ranging from mild swelling to gross irreversible inflammatory damage and cell death.

1.12. Treatment of post-streptococcal CNS disease

1.12.1. Penicillin prophylaxis

There are two potential treatment approaches to post-infectious autoimmune disorders: either to prevent further exacerbating infections or to modulate the autoimmune process. Antibiotic prophylaxis is an accepted treatment of RhF and SC, and should be continued throughout childhood (Dajani AS et al., 1989). It should be noted that large studies were required to establish a benefit of penicillin prophylaxis in SC. Initial attempts to replicate these findings in PANDAS using a double blind placebo controlled trial were unsuccessful, although this may have been due to a failure to achieve acceptable levels of prophylaxis (Garvey MA et al., 1999). A prospective study of antibiotic treatments for acute tonsillitis in PANDAS showed prompt improvements in obsessive-compulsive behaviours, although this was an uncontrolled descriptive study (Murphy ML et al., 2002).

A recent double-blind randomised controlled study examining penicillin and azithromycin prophylaxis in PANDAS patients (total n=33) demonstrated significant reduction in GAS infections in both penicillin and azithromycin groups, therefore demonstrating effective prophylaxis. More importantly, the treatment arms had significant reduction in neuropsychiatric exacerbations compared to the prior untreated year (Snider LA et al., 2005).

1.12.2. Immunomodulation therapies

1.12.2.1. Sydenham's chorea

There have been no formal studies of intravenous immunoglobulin (IVIG) or plasma exchange in SC. One study with few numbers (n=9: five plasma exchange, four IVIG) was published as a preliminary paper (Garvey MA et al., 1996). Both treatment arms improved although the plasma exchange group improved very rapidly, whereas three of the four IVIG treated children relapsed within four months of completing

treatment. Plasma exchange was held as the superior therapy. Small studies have examined the effectiveness of steroids in the treatment of SC. One retrospective study of eight patients showed rapid improvements in their treated patients (Green LN, 1978). A further study described SC cases refractory to routine therapy (valproate and neuroleptics) who were treated with intravenous methyl-prednisolone and then oral prednisolone. Five patients were treated and the authors concluded that these treatments were effective in refractory SC (Cardoso F et al., 2003). A study published only in abstract form from Utah in the mid-1980's (Thompson JA et al., 1999) compared 36 prednisolone treated patients with 22 untreated childrem. The median time to recovery was 2.75 weeks in the prednisolone group compared to 10 weeks in the untreated group.

1.12.2.2. PANDAS

The only placebo controlled trial examining the benefit of immunomodulation (plasma exchange and intravenous immunoglobulin) in PANDAS demonstrated improvements in the patients treated with active agents compared to patients treated with sham (saline) infusions. Importantly, the treatment improvements were maintained at one year (Perlmutter SJ et al., 1999). Interestingly, the same finding was not reproduced in OCD patients who did not have PANDAS, suggesting that the benefit of immune modulation is restricted to the PANDAS subgroup of neuropsychiatric disorders (Nicolson R et al., 2000).

1.12.3. Conventional therapies and summary of treatments

Currently, immune treatments should not be given routinely to SC or PANDAS patients until further controlled trials confirm their benefit. Carbamazepine and

sodium valproate have been proposed to be useful symptomatic treatments of SC, and are probably preferable to haloperidol, which can cause unacceptable side effects (Pena J et al., 2002).

In summary, at present it is probably best to recommend symptomatic treatment in the active phase rather than potentially toxic immune modulating therapies. Penicillin or azithromycin prophylaxis is recommended in SC and looks promising in PANDAS, particularly given the good side effect profile of antibiotic prophylaxis.

1.13.Clinical aims of this thesis

The clinical aims of this thesis are to:

1. Define the spectrum of post-streptococcal extrapyramidal movement disorders in a tertiary referral sample in the UK.
2. To define the psychiatric co-morbidity in post-streptococcal extrapyramidal movement disorders.
3. Review the neuroimaging in post-streptococcal neuropsychiatric syndromes.

Chapter 2. Methods- Patient recruitment of post-streptococcal neuropsychiatric disease

2.1 Movement disorders

2.1.1 Movement disorders: characteristics and terminology

Movement disorders describe abnormal or extra involuntary movements. The term 'movement disorders' usually infer extrapyramidal movements, although cerebellar ataxia has sometimes been included in the 'movement disorder' spectrum. However, in the context of this work the term 'movement disorders' will infer extrapyramidal movement disorders alone. Extrapyramidal movement disorders are classified broadly into two main groups;

1. hypokinetic-rigid syndromes (paucity of movements)
2. hyperkinetic movement disorders or dyskinesias (extra or excessive movements)

The hypokinetic-rigid syndromes (commonly referred to as Parkinsonism) require little further classification. Hypokinetic-rigid syndromes describe bradykinetic movements and/or akinesia, tremor (classically at rest) and rigidity. However, the dyskinetic movement disorders require further classification according to a number of characteristics, namely:

I. Rhythmicity of extra movements

II. Speed of movements

III. Stereotypical nature of movements

Using these further criteria, it is possible to characterise the dyskinesias into the following terminologies:

A. Chorea and ballismus

B. Dystonia

C. Tremor

D. Myoclonus

E. Tics

The following table describes the different dyskinetic movement disorders according to their characteristics:

TABLE 2.A. CHARACTERISTICS OF DIFFERENT MOVEMENT DISORDER PHENOTYPES

Characteristic	Chorea	Dystonia	Tremor	Myoclonus	Tics
Rhythmicity	No	No	Yes	Either	No
Speed	Fast	Slow and sustained	Fast	Fast	Fast
Stereotypical or variable	Variable	Stereotypical	Stereotypical	Stereotypical	Stereotypical
Suppressible	No	No	No	No	Yes
Other features	'Dance-like'	Twisting	Repetitive	Often regional	Preceded by an urge or sensation

Ballismus is related to chorea, but typically involves high velocity displacement, often involving the limbs.

It is has been recognised for years that different clinicians may classify the same movement disorder in different ways. Being aware of this, I videoed movement disorders of 16 patients, some of which were difficult to classify. I then performed an inter-rater comparison of movement disorder classification.

2.1.2. Movement disorder classification: Inter-rater assessment

Method

I aimed to examine possible differences in classification between raters. The 16 videos featured a range of extrapyramidal movement disorders in patients with post-streptococccal neuropsychiatric disease. The videos were presented in digital form on computer. The raters filled in the forms independently and simultaneously. They were asked to classify the primary movement disorder and secondary movement disorder where two or more movement disorders co-existed.

The raters were:

1. Dr Russell Dale, Primary investigator (RCD)
2. Professor Robert Surtees, Professor of Paediatric neurology (RAHS)
3. Professor Brian Neville, Professor of Paediatric neurology (BGRN).

Results

As can be seen in table 2.b, there was general agreement on the classification of these 16 videos. Where total agreement was absent, the three raters agreed upon a final movement disorder classification for each patient that was used in the clinical results.

Table 2.b. Videos of 16 patients with movement disorders rated by 3 physicians.

Video (initials)	RCD	BGRN	RAHS
JW	Paroxysmal dystonia and chorea	Chorea and dystonia	Paroxysmal dystonia and chorea
CN	Hemiballsimus Hemidystonia	Hemiballismus	Hemiballismus, Parkinsonism and hemidystonia
GH	Rest tremor	Coarse tremor, extrapyramidal	Dystonic or rubral tremor
MO	Dystonia Blepharoclonus	Dystonia, Parkinsonism	Parkinsonism and blepharospasm
TT	Parkinsonism	Parkinsonism	Parkinsonism
LW	Dystonia and chorea	Dystonia and chorea	Dystonia and chorea
AA	Chorea	Dystonia and chorea	Choreiform
SB	Chorea	Chorea	Chorea
CR	Chorea Complex tics	Tics	Complex tics
FA	Stereotypies	Stereotypies	Tics
JG	Tics	Complex tics	Tics
SK	Chorea	Chorea	Chorea (distal)
CI	Myoclonic tics	Myoclonic dystonia	Chorea and dystonia
HS	Chorea Opsoclonus and myoclonus	Opsoclonus, ataxia	Dystonia Opsoclonus and myoclonus
HT	Dystonia and chorea	Dystonia	Dystonia and ataxia
JT	Tremor, Parkinsonism	Tremor, Parkinsonism	Dystonia

2.2 Psychiatric assessments

As a paediatric neurologist, despite a lack of formal training in psychiatry, I needed to be able to produce internationally acceptable psychiatric diagnoses in patients with post-streptococcal neuropsychiatric syndromes. After discussion with Professor Robert Goodman, Professor of Child Psychiatry, Institute of Psychiatry, London, it was suggested that the Development and Well Being Assessment (DAWBA) would be the most appropriate interview.

2.2.1 The Development and Well Being Assessment

The DAWBA is an integrated package of measures for child psychiatry that has been validated in clinic and community samples for 5-16 year olds. It is performed with the parent or individual (over 11 years old). The interview can be performed by non-clinical interviewers using a structured interview in a computer-assisted diagnostic rating package. The DAWBA interviews and questionnaires were provided by Robert Goodman in a Microsoft Access package (Goodman R et al., 2000). The interview covers specific areas in detail: separation anxiety, generalised anxiety, obsessive-compulsive disorder, major depression, specific and social phobias, post-traumatic stress disorder, hyperkinesis/ADHD, and conduct-oppositional disorders. The interview asks about all symptoms required for operationalised diagnoses using both DSM-IV (American Psychiatric Association, 1994) and ICD-10 (World Health Organisation, 1994). Certain disorders are only asked about briefly, namely panic disorder, agoraphobia, autistic disorders, tic disorders and eating disorders, and would depend upon the open-ended transcript (discussed shortly). The symptoms relate to present and recent past. Importantly, the interview focuses upon the impact of these

symptoms and associated morbidity, an essential part of making an internationally acceptable psychiatric diagnosis. The computer-generated diagnoses are based upon computerised diagnostic algorithms. These diagnoses are not definitive, but provide a useful start for the expert clinical raters. To improve validity and check that the questions were fully understood, the problems are described in the patient's own words. The expert clinical rater reviews this open text. In addition, for further validation, a separate questionnaire is performed, namely the Strengths and Difficulties questionnaire (SDQ). The SDQ can generate emotional, conduct and hyperactivity scores to support the DAWBA generated diagnoses. The SDQ presents a brief measure of the adjustment and psychopathology of children and adolescents (Goodman R, 2001). Once the information has been added to the computer package (DAWBA, SDQ and written text), the data was saved and given to the clinical rater. For this study, the clinical rater was Dr Isobel Heyman, Consultant child psychiatrist, Institute of Psychiatry, London. Dr Heyman is an experienced child psychiatrist, who has received training in order to evaluate these interviews. The raters check whether the respondents have fully understood the questions, and ensure that the computer-generated diagnoses conform to the written text and SDQ scores. In borderline cases, consensus diagnoses where made with the help of Robert Goodman. The DAWBA has been validated by comparing the usefulness of the interview in clinic and community cohorts. The DAWBA diagnoses correlated well with the clinical case note diagnoses (Goodman R et al., 2000). In addition, the DAWBA was used in an extremely large population-based study of psychiatric (DSM-IV) diagnoses in British children. In this study, 10,438 children were assessed using the DAWBA. The findings of this study are summarised in table 2.c, and provided an important control group to compare with the post-streptococcal neuropsychiatric patients. In table 2.c,

the ICD-10 criteria are listed (Meltzer H et al., 2000), although there is a strong correlation between ICD-10 and DSM-IV criteria (Ford T et al., 2003).

Table 2.c. The ICD-10 diagnoses of 10,438 children from a population-based sample.

Diagnosis	Point prevalence ICD-10
Any psychiatric diagnosis	8.9%
Emotional disorder	4.3%
Obsessive-compulsive disorder	0.2%
Generalised anxiety	0.6%
Depressive episode	0.7%
Separation anxiety	0.8%
Specific phobia	1.0%
Social phobia	0.3%
Panic attacks	0.1%
Conduct disorders	4.7%
Oppositional defiant disorder	2.5%
Other conduct disorders	2.2%
Hyperkinetic disorders	1.3%
Hyperkinesis	1.1%
Other hyperkinetic disorder	0.2%
Less common disorders	0.5%

2.3 Clinical and laboratory diagnosis of Streptococcal infection

Group A streptococcus (GAS) infections are common in children. Most commonly they cause pharyngeal infections, presenting as sore throat, lymphadenopathy and

occassionally systemic symptoms. It is also recognised that GAS can reside in the pharynx without producing local inflammation (i.e. carriers).

As GAS infections are common, markers of GAS infection are frequently elevated in paediatric populations, more so than adult populations. In view of this, I needed to use clear clinical and laboratory criteria for including patients into the post-streptococcal neuropsychiatric cohort.

Method

To make a diagnosis of post-streptococcal neuropsychiatric disease, I based the criteria upon previously described diagnostic criteria from Sydenham's chorea (Special writing group of the committee on rheumatic fever, 1992) and PANDAS (Swedo SE et al., 1998).

1. The patient is a previously well child with acute or subacute onset of neurological or psychiatric disease occurring shortly (within 2 months) of a pharyngeal infection compatible with GAS. Alternatively, if the patient already has a neuropsychiatric syndrome, there must be two or more relapses or deteriorations associated with GAS infection.
2. The infection should be compatible with GAS pharyngeal infection. This should include the following symptoms: sore throat, cervical lymphadenopathy, fever, and abdominal pain (at least 2 of these).
3. All patients had evidence of recent or current GAS infection. Laboratory evidence of GAS should be either:
    a. Microbiological culture of GAS or beta haemolytic streptococcus species from the throat.
    b. Positive streptococcal serology (ASOT or anti-DNase B) acutely with a reduction in titre on convalescent testing (3-6 months).

4. No other explanation for the neurological or psychiatric syndrome.

Other markers of a post-infectious immune mediated syndrome were also measured, and the results are reported in the results including:

- Erythrocyte sedimentation rate
- Cerebrospinal fluid cells, protein, oligoclonal bands when performed
- MRI brain. All MRI brain scans were performed on a 1.5 Tesla magnetic resonance scanner. The standard T1 and T2 weighted images were acquired in the coronal and sagital planes. All images were reported by consultant neuroradiologists.
- Echocardiography and electrocardiography

These further investigations were performed according to the discretion of the consulting physician and were not performed in all patients.

2.4 Patient recruitment

The patients were all recruited via Great Ormond Street hospital NHS trust neurosciences unit. The patients presented with movement disorders or neurological syndromes after streptococcal infection. Some patients were recruited through the routine neurology service. Others were referred directly to RCD from other centres, and were subsequently seen as in-patients or out-patients at Great Ormond Street hospital NHS trust. All patients were interviewed as follows:

1. Past medical history and family history of neurological, psychiatric and autoimmune disorders.
2. History of preceding infection.

3. History of neurological syndrome. Atypical or unusual movement disorders were videoed for further analysis as described before. Movement disorders were classified according to accepted criteria.
4. Psychiatric interview as described above: DAWBA, SDQ and written text.
5. Symptom review for other symptoms.
6. Clinical examination for neurological and systemic signs.
7. Laboratory investigation as described above. Additional investigation to exclude other causes of the neuropsychiatric syndrome.

2.5 Statistics used in clinical characteristics and control groups

Clinical differences between patient groups and controls/ normative data were examined using chi-squared test, Fisher's test or the Mann Whitney test as appropriate. Anti-streptolysin-O and anti-DNase B titres in patients and controls were analysed using the non-parametric Mann Whitney test.

Control groups used in this thesis were all recruited from Great Ormond Street hospital NHS Trust, or the National Hospital for Neurology and Neurosurgery. All were consented for this investigation. The following control groups were recruited:

- Child neurology control group (n=100, mean age 8.2, range 1-16 yrs)
- Child Streptococcal control group- all had recent infection compatible with streptococcal infection but no neurological disease, plus positive streptococcal serology (n=40, mean age 9.8, range 2-15 yrs).
- Child autoimmune control group (n=50, mean age 9.2, range 2-16 yrs)
- Adult neurology group- random neurological controls (n=50, mean age 41, range 19-70 yrs)
- Adult healthy group (n=50, mean age 35.6, range 19-57 yrs)

Chapter 3. Results- the clinical spectrum of post-streptococcal neuropsychiatric disease

3.1 Introduction

The patients in this chapter will be presented as follows:

- Post-streptococcal CNS patients
  1. Dyskinesia
  2. Acute disseminated encephalomyelitis (ADEM)
  3. Parkinsonism

- Encephalitis lethargica, of whom a proportion were post-streptococcal.

3.2 Post-streptococcal dyskinesias (n=40)

3.2.1. Definition of patients

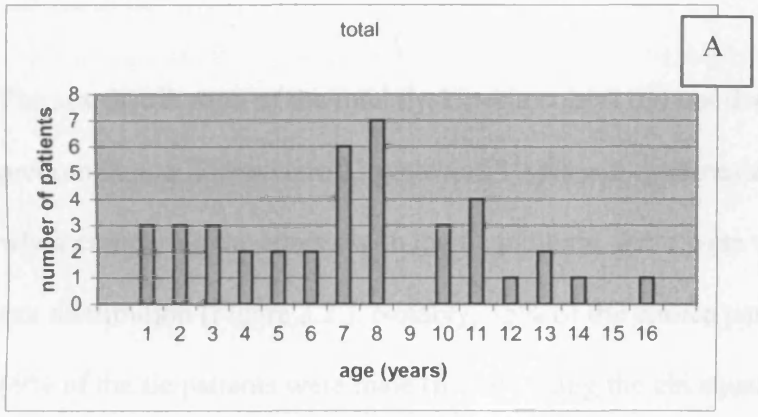
Patients were referred between 1999-2002 by their general practitioner or paediatrician for investigation and management of an acute onset or relapsing dyskinetic movement disorder. All patients included had disease onset shortly after streptococcal pharyngeal infection, or suffered two or more relapses after streptococcal pharyngeal infections (Swedo SE et al., 1998). Evidence of Group A streptococcal (GAS) infection was evident in all patients, and was diagnosed when a clinical episode of pharyngitis occurred with laboratory evidence of GAS infection (growth of GAS organism on pharyngeal swab, or elevated acute streptococcal serology with reduction in serology 3-6 months later). Investigations for an alternative cause of chorea, dystonia and myoclonus (copper, caeruloplasmin, anti-nuclear antibody, thyroid function tests, lactate, plasma amino acids and urine organic acids) were normal in all patients.

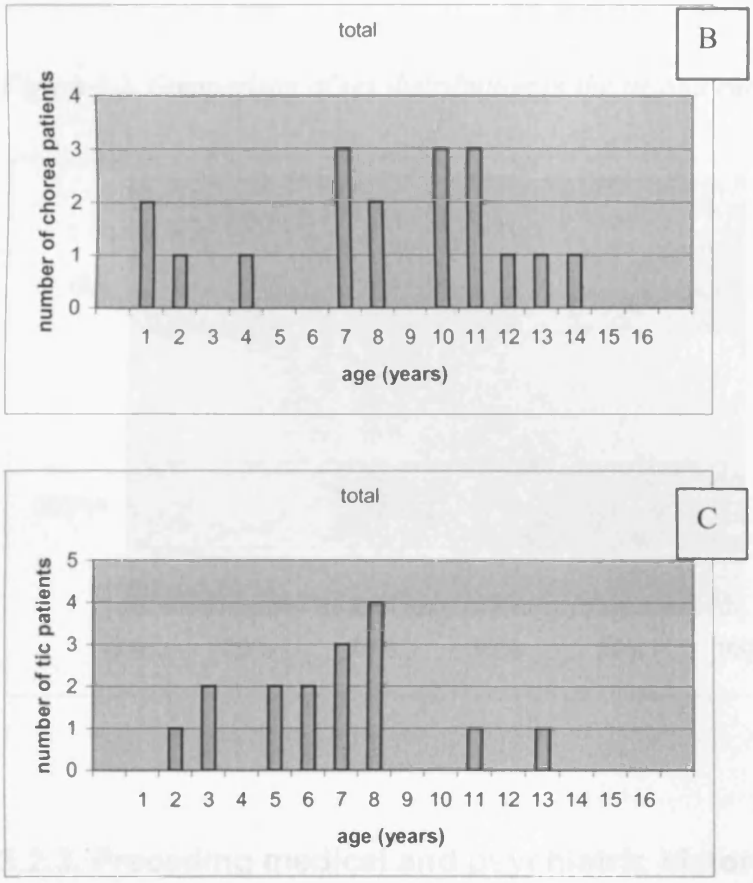
3.2.2 Demographics

3.2.2.1. Age

The age range of movement disorder onset was 1.2 –16 years (mean 7.3 years, median 7 years). The breakdown of patient age according to the movement disorder phenotype is presented in figure 3.1.a. As can be seen, the total cohort is distributed widely over the whole of the paediatric range, with a peak in the 5-10 year age group. The chorea patients were more likely to be older (Figure 3.1.b. (mean 7.85 yr, median 8 yr) than the tic patients (Figure 3.1.c. (tic group mean age 6.75 yr, median 7 yr), although this difference did not reach statistical significance.

Figure 3.1. Age distribution of total dyskinesia cohort (A), the chorea subgroup (B) and the tic subgroup (C).

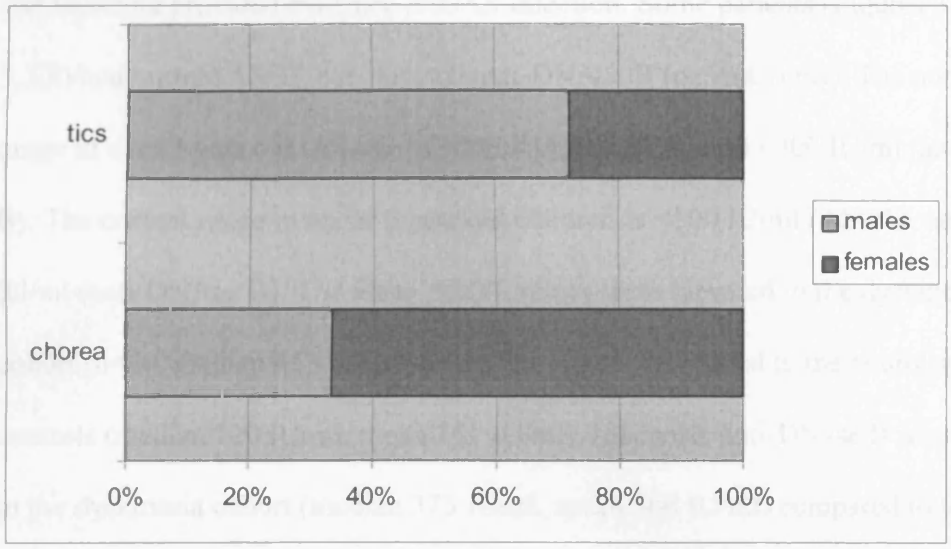




3.2.2.2. Sex

The sex distribution of the total dyskinesia cohort did not demonstrate clear sex predominance. There were 21 male and 19 female dyskinesia patients. However, when comparing the chorea with the tic patients, there were some differences in the sex distribution (Figure 3.2.). Notably, 35% of the chorea patients were male, whereas 69% of the tic patients were male (p=0.04 using the chi squared test).

Figure 3.2. Comparison of sex distribution in the tic and chorea subgroups.



3.2.3. Preceding medical and psychiatric history

Psychiatric symptoms were present before movement disorder onset in seven patients (17.5%): attention deficit hyperactivity disorder n=6 (one with co-morbid OCD and generalised anxiety), and post-traumatic stress disorder n=1. Other past medical history included cows milk protein allergy (n=1), asthma (n=1) and moya-moya disease (n=1).

3.2.4 Precipitating infectious illness

Thirty-four patients (85%) had an infectious illness compatible with GAS shortly before movement disorder onset. The remaining six patients did not have a clinical history of GAS infection at movement disorder onset, but subsequently had two or more relapses associated with streptococcal infections. GAS organisms were grown

from pharyngeal cultures in six patients (three with chorea, three with tics). Streptococcal serology (ASOT and anti-DNAse B) was elevated in all other patients and therefore provided evidence of GAS infection. Some patients (Figures 3.3.a and 3.3.b) had normal ASOT but elevated anti-DNAse B (or visa versa). The normal range in over 3 year old children is <200 IU/ml (ASOT) and <300 IU/ml (anti-DNAse B). The normal range in under 3 year old children is <100 IU/ml (ASOT), and <200 IU/ml (anti-DNAse B). The mean ASOT values were elevated in the dyskinesia cohort (n=40, median 453 IU/ml, mean 529 IU/ml) compared to the neurology controls (median 100 IU/ml, mean 151 IU/ml). Likewise, anti-DNase B was elevated in the dyskinesia cohort (median 373 IU/ml, mean 566 IU/ml) compared to the neurology control groups (median 196 IU/ml, mean 213 IU/ml) (Figures 3.3.a and 3.3.b). The mean latency between infection and movement disorder onset was 18.9 days (range 1-67 days).

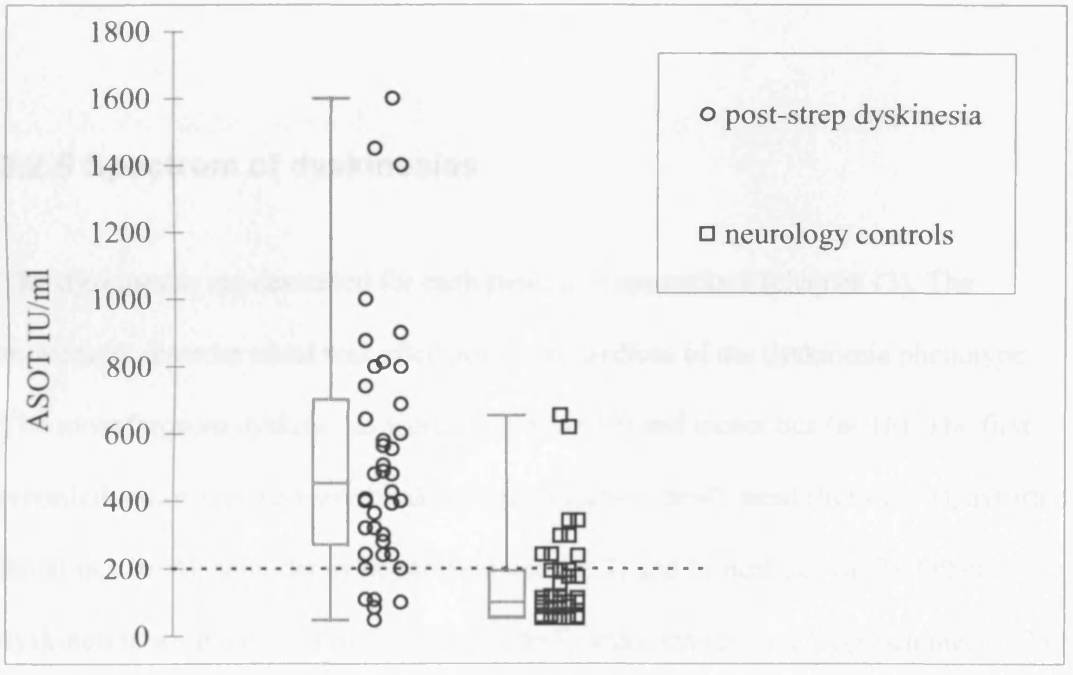


Figure 3.3.a. *ASOT in post-streptococcal dyskinesia group and neurological controls. Bars presented are 0th, 25th, 50th, 75th and 100th centiles. Dotted line represents internationally defined significant cut-off (ASOT>200IU/ml).*

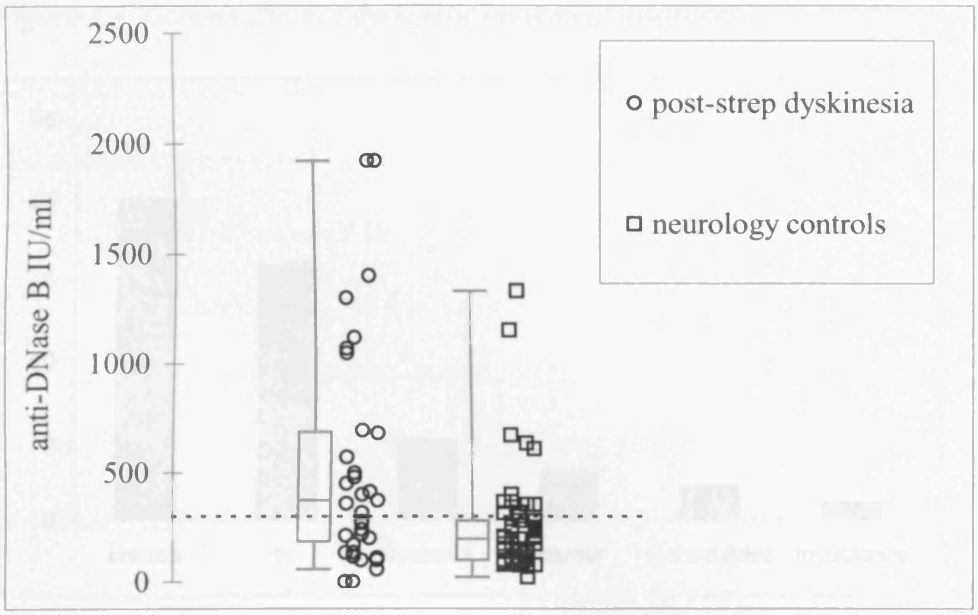
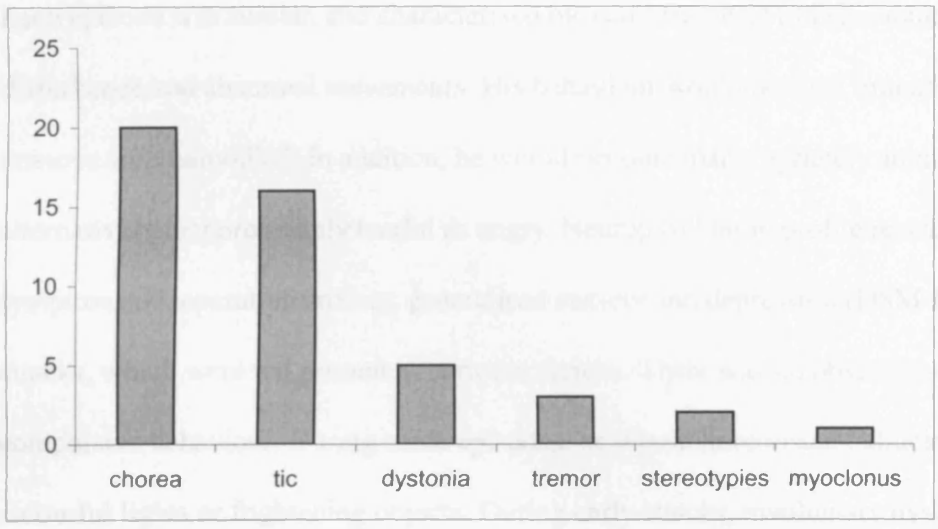


Figure 3.3.b. *Anti-DNase B in post-streptococcal dyskinesia group and neurological controls. Bars presented are 0th, 25th, 50th, 75th and 100th centiles. Dotted line represents internationally defined significant cut-off (anti-DNAse B>300IU/ml).*

3.2.5 Spectrum of dyskinesias

The dyskinesias are described for each patient in appendix 1 (chapter 13). The movement disorder onset was often abrupt, regardless of the dyskinesia phenotype. The most frequent dyskinesias were chorea (n=20) and motor tics (n=16). The first recorded motor tics were eye blinking/eye deviation (n=4), head flicks (n=3), assorted facial tics (n=4), shoulder or upper limb tics (n=3) and truncal tics (n=2). Other dyskinesias were dystonia (n=5), tremor (n=3), stereotypies (n=2), opsoclonus (n=2) and myoclonus (n=1). The dyskinesia patients can be summarised with the following pie chart (Figure 3.4).

Figure 3.4. Column chart of dyskinetic movement disorders.



Seventeen patients had one or more vocal tics; 13 associated with motor tics, two with chorea, one with myoclonus and one with stereotypies.

3.2.5.1. Notable cases of post-streptococcal dyskinetic movement disorders

There were a few cases that were notable because of their presentation:

Case 1

An eight year old boy had been previously well other than recurrent upper respiratory tract infections. There was no personal or family history of neuropsychiatric disease or movement disorder. One week after a sore throat, he presented with an acute onset behavioural disturbance and abnormal 'jumpy' movements. The episode lasted two hours and resolved completely. From this time, he suffered between 1-4 paroxysmal attacks per day. Each attack would start suddenly. Attacks were not precipitated by

movement nor exercise, although stress and anxiety increased the attack frequency. Each episode was similar, and characterised by a sudden onset of behavioural disturbance and abnormal movements. His behaviour would become immature, anxious and 'baby-like'. In addition, he would become inappropriately animated, or alternatively inappropriately tearful or angry. Neuropsychiatric profile revealed symptoms of separation anxiety, generalised anxiety and depression (DSM-IV) during attacks, which were not present in between attacks. There was no obsessive-compulsive behaviour. During some episodes, he would have visual hallucinations of colourful lights or frightening objects. During early attacks, involuntary dystonic posturing of his hands was observed. As the events evolved there was dystonic posturing of the face, head, limbs and trunk, with choreoathetosis becoming evident towards the end of an attack. During an event, he would be unable to walk unaided, and attempts to weight bear resulted in continuous dancing movements of the legs resembling St Vitus dance. These movements were not evident when he was lying supine on the bed. Each attack would last between 10 minutes and 4 hours, and there would be 1-4 attacks per day. In between attacks, his behaviour and neurological examination was normal.

MRI brain imaging and EEG during an attack was normal. The following investigations were normal or negative: full blood count, erythrocyte sedimentation rate, c-reactive protein, antinuclear antibody, autoantibody profile, thyroid function, urate, lactate, caeruloplasmin, copper, plasma amino acids, urine toxicology and echocardiogram. Anti-streptolysin-O titre was elevated to 406 IU/ml (normal <200 IU/ml) and anti-DNase B titre 256 IU/ml (normal <300 IU/ml). Convalescent serology showed a reduction in titres.

A post-streptococcal neuropsychiatric syndrome was suspected and penicillin prophylaxis was commenced. Over the following 2 months, he continued to have

paroxysmal events, occurring up to 4 times per day as described. His gait was so impaired he needed to use a wheelchair. A trial of chlorpromazine therapy had no impact on the attacks, and caused unacceptable drowsiness. By the 3rd month, the paroxysmal attacks became less frequent, occurring only on alternate days. He only had 2 events in the 4th month of his illness. In the 5th month, he had an exacerbation in attack frequency associated with the onset of hayfever. In the 6th month he was started on carbamazepine in an attempt to reduce paroxysmal events, which has halved the number of attacks in the first month of therapy. He remains on penicillin prophylaxis.

Case 2

A male infant born at term by uncomplicated delivery had achieved normal developmental milestones. At 14 months of age, he could walk unaided, feed himself and was vocalising keenly. There was no family history of neurological disease. At 14 months, he suffered a seven day febrile illness with signs of upper respiratory tract infection. He recovered uneventfully but 10 days later had an acute neurological illness. Examination at this time demonstrated asymmetric motor weakness (left more than right), truncal ataxia and dystonic posturing. Within 3 days, he was spontaneously improving and returned back to normal within one week. However, seven days later he suffered a further episode of pharyngitis. This was immediately followed by a rapid decline in his function. Over the following two weeks, he lost the ability to walk, crawl and sit. Examination demonstrated an upper limb resting tremor, rigidity and dystonic posturing of the arms and legs. He had oro-pharyngeal incoordination resulting in drooling and feeding difficulties. His condition stabilised for four weeks, but then deteriorated further associated with another upper respiratory tract infection. At this stage symmetrical dystonia persisted with new oro-facial

grimacing and generalised chorea. His behaviour, sleep pattern and alertness remained normal throughout.

MRI brain revealed abnormalities that were restricted to the basal ganglia. There was bilateral caudate and putaminal enhancement on T2 weighted sequences. Anti-streptolysin O titres were 197 IU/ml acutely and 57 IU/ml on convalescent testing (normal range in this age group <100 IU/ml). These findings were consistent with previous streptococcal infection. The following investigations were normal or negative; full blood count, c-reactive protein, CSF microscopy and culture, CSF polymerase chain reaction for herpes simplex virus, varicella zoster virus, cytomegalovirus, enterovirus, echovirus, human herpes virus 6 and human herpes virus 7. Blood serology was also negative for mycoplasma, adenovirus, influenza A and B, enterovirus and chlamydia. Urine, throat and blood cultures were negative. The following metabolic investigations were negative or normal; copper, caeruloplasmin, lactate (blood and CSF), CSF glucose, liver function tests, very long chain fatty acids, lysosomal enzymes, acylcarnitines, amino acids, urinary organic acids, ammonia, creatine kinase, transferrin isoforms and lipid electropheresis. Ophthalmology, EEG, electroretinogram and visual evoked potential were all normal. CSF protein was elevated at 0.8 g/dl.

A post-streptococcal autoimmune neuropsychiatric disease was suspected. He was started on Penicillin V prophylaxis. Two mg/kg oral prednisolone was also commenced for two weeks followed by a six week weaning regimen. Over the following two months, in conjunction with physiotherapy and occupational therapies, he made significant functional improvements. The rigidity reduced, and his dystonia was less pronounced. Involuntary writhing movements of the upper limbs and trunk persisted but were less impairing. Assessment at 10 months (aged two years) showed

residual dystonia and chorea. Functionally, he was able to sit unsupported but still unable to walk.

These two cases were notable for the following reasons. Case 1 presented as paroxysmal dystonic choreathetosis, a previously undescribed phenotype of post-streptococcal neuropsychiatric disease. Case 2 presented with acute onset dystonia and basal ganglia lesions, in a manner previously termed 'infantile bilateral striatal necrosis'. Both of these cases were subsequently reported in the medical literature, being examples of the expanding spectrum of post-streptococcal neuropsychiatric disease (Dale RC et al., 2002; Dale RC et al., 2002).

3.2.6 Psychiatric comorbidity

The psychiatric comorbidity is described for each patient in appendix 1 (chapter 13). Acute emotional and/or behavioural alteration occurred in 33 patients (82.5%). Most frequently reported acute changes were emotional lability (n=13, 32.5%), anxiety (n=11, 27.5%), obsessions and/ or compulsions (n=9, 22.5%), and depression (n=7, 17.5%). Other common behavioural changes included aggressive, oppositional or disruptive behaviours (n=14, 35%) and attention deficit (n=11, 27.5%). Less common psychiatric manifestations included echolalia (n=4), visual hallucinations (n=2), and social disinterest (n=2). Formal psychiatric assessments were carried out at least two weeks after the acute presentation. One or more ICD-10 diagnoses were made in 25 patients (62.5%). Thirteen patients (32.5%) had 2 or more psychiatric diagnoses. The ICD-10 diagnoses are presented in Table 3.1.with comparison to previously derived normative data (Meltzer H et al., 2000). As can be seen, emotional disorders were the most frequent psychiatric phenotype, of which obsessive-compulsive disorder was the

most common, followed by other anxiety disorders and major depression. Conduct and hyperkinetic disorders occurred less frequently than emotional disorders, although significantly more commonly than would be expected in a normal population (Table 3.a.). Due to low numbers in some psychiatric comorbidity groups, statistical analysis was only applied when the number of patients with the psychiatric disorder was greater than, or equal to 5 (12.5%).

Table 3.a. Psychiatric diagnoses in post-streptococcal dyskinesia cohort (n=40) and national comparison group (n=10,438) (Meltzer H et al., 2000).

ICD-10 diagnosis	Point prevalence		P
	Dyskinesia group	Normative data	(continuity -adjusted chi-square)
Any ICD-10 diagnosis	**62.5%**	**8.9%**	**.000**
Emotional disorder	**47.5%**	**4.3%**	**.000**
Obsessive-compulsive disorder	27.5%	0.2%	.000
Generalised anxiety	25.0%	0.6%	.000
Depressive episode	17.5%	0.7%	.000
Separation anxiety	7.5%	0.8%	
Specific phobia	7.5%	1.0%	
Social phobia	5.0%	0.3%	
Panic attacks	2.5%	0.1%	
Conduct disorders	**27.5%**	**4.7%**	**.000**
Oppositional defiant disorder	15.0%	2.5%	.000
Other conduct disorders	12.5%	2.2%	.000
Hyperkinetic disorders	**15.0%**	**1.3%**	**.000**
Hyperkinesis	12.5%	1.1%	.000
Other hyperkinetic disorder	2.5%	0.2%	
Less common disorders	**10%**	**0.5%**	

3.2.7 Other clinical features

3.2.7.1. Other neurological features

Hypotonia and bulbar dysfunction were common in the chorea subgroup (dysarthria occurred in half of the chorea patients). Sleep disorder occurred in nine patients (22.5%), particularly insomnia during the acute phases. Less common features included reduced consciousness (n=2), epileptic seizures (n=2), and mutism (n=2).

3.2.7.2. Systemic features

Systemic complications occurred in nine patients (22.5%), and affected the chorea patients only (47.5% of chorea patients had systemic features); five had carditis defined using echocardiography (four with mitral regurgitation). Two patients had arthritis, and three other patients had arthralgia. One further patient had a vasculitic rash, but no patients had erythema marginatum. Systemic complications always preceded the neurological syndrome.

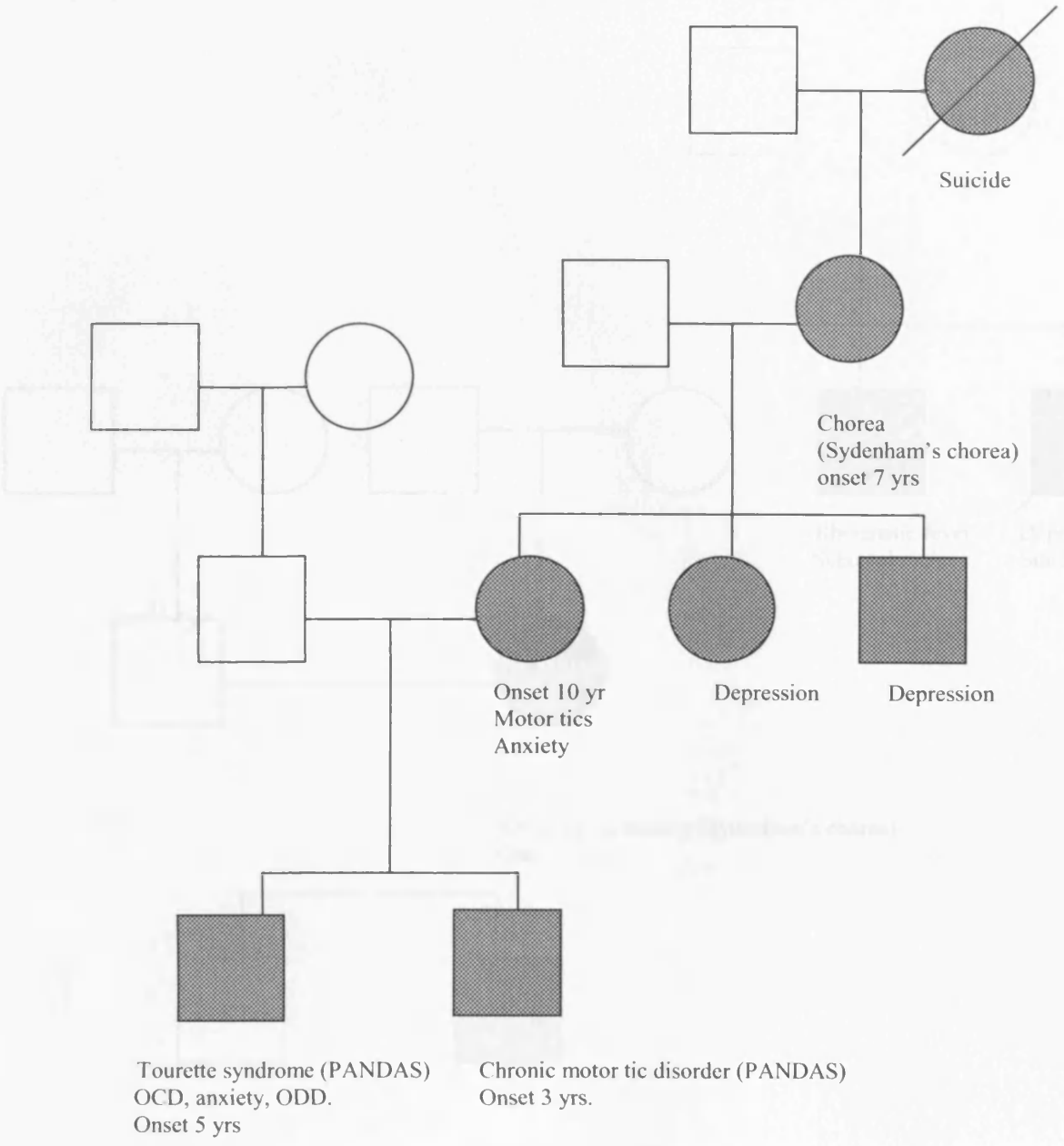
3.2.8 Family history

Sixteen patients (40%) had a family history of psychiatric or movement disorders in 1st degree family members. Ten patients (25%) had at least one family member with a history of movement disorders; motor tics in childhood (n=7), Sydenham's chorea (n=2) and Tourette's syndrome (n=1). Family history of psychiatric disorders (ICD-10) was present in eleven patients (27.5%); obsessive-compulsive disorder (n=5), depression (n=4), hyperactivity (n=3), anxiety, conduct disorder and chronic fatigue syndrome (all n=1) (some family members had more than one psychiatric diagnosis).

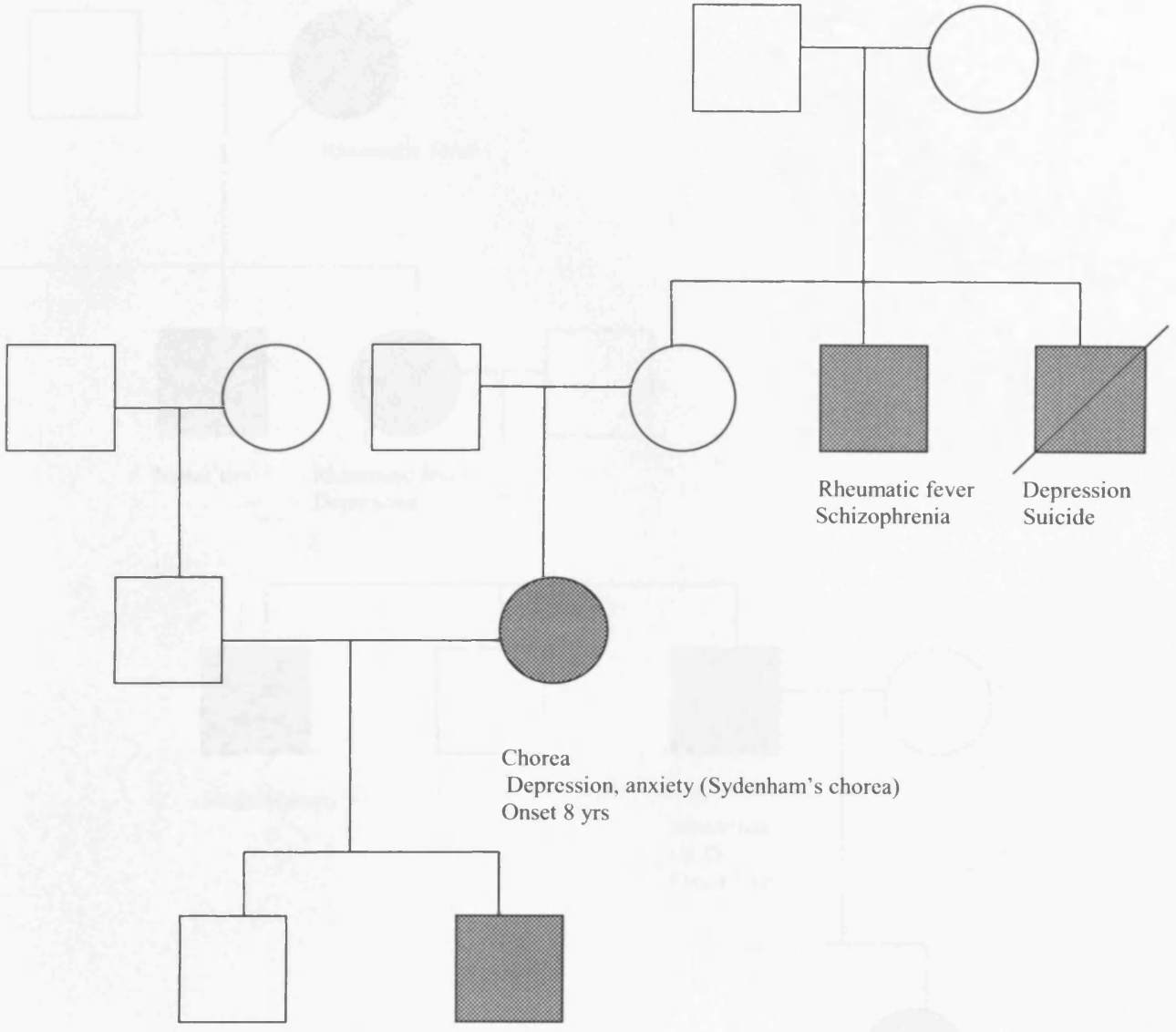
A family history of post-streptococcal autoimmune complications in 1st or 2nd degree family members was present in eight patients (20%); Sydenham's chorea (n=3), PANDAS (n=3) and rheumatic carditis (n=2). Some families exhibited very strong family histories of autoimmune, movement and psychiatric disorders as demonstrated in figures 3.5..

Figure 3.5. *Family trees. Post-streptococcal diagnosis is listed in brackets (when present). OCD: Obsessive-compulsive disorder. ODD: Oppositional defiant disorder.*

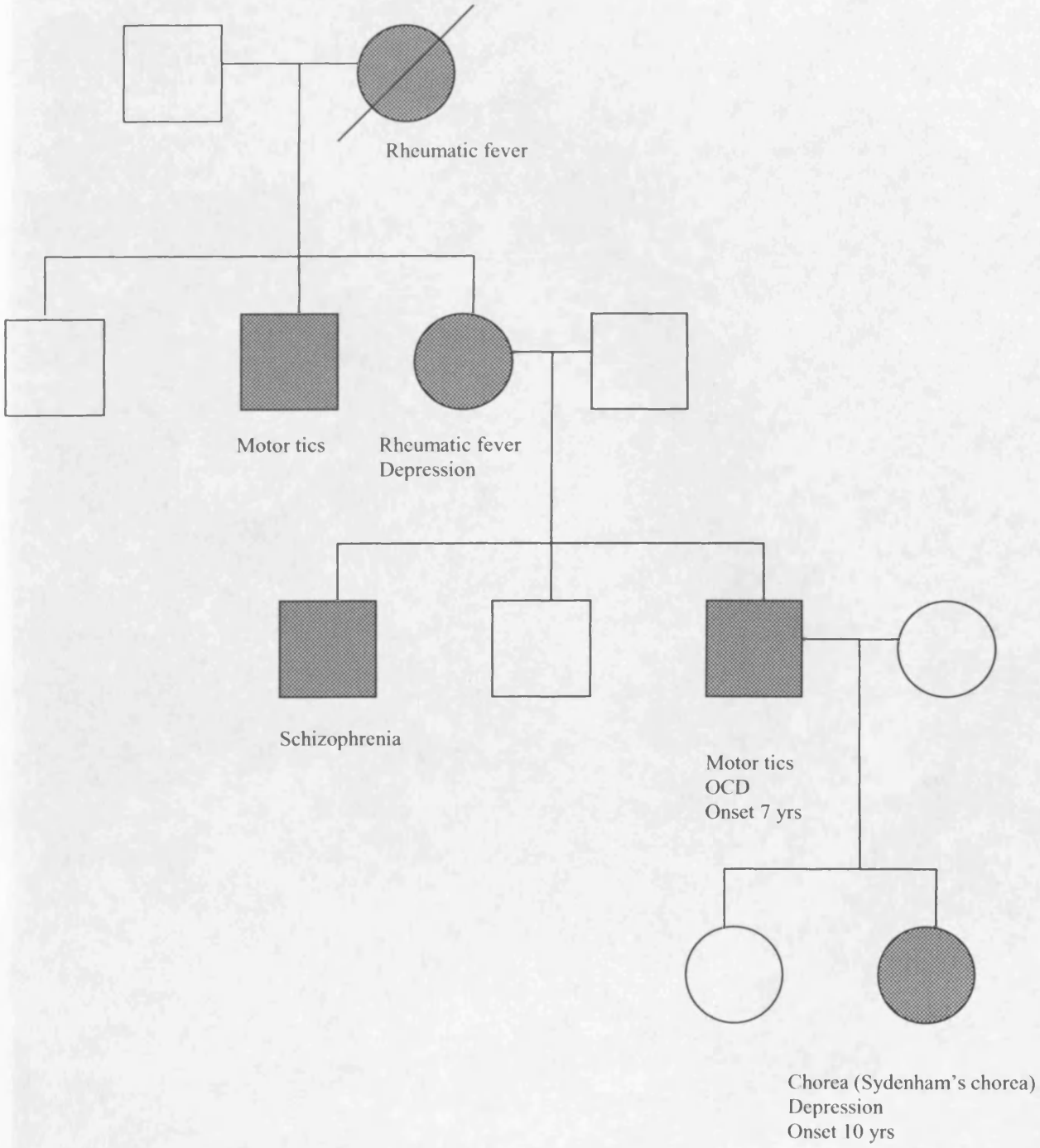
Family 1



Family 2


Rheumatic fever
Schizophrenia
Depression
Suicide
Chorea
Depression, anxiety (Sydenham's chorea)
Onset 8 yrs
Transient tic disorder
OCD, anxiety, depression (PANDAS)
Onset 13 yrs

Family 3


Rheumatic fever
Motor tics
Rheumatic fever
Depression
Schizophrenia
Motor tics
OCD
Onset 7 yrs
Chorea (Sydenham's chorea)
Depression
Onset 10 yrs

3.2.9 Clinical outcome

Patients with persistent disease are still under medical care. The mean duration of disease in this cohort is currently 2.7 years (2 months-13 years). Eleven (27.5%) patients have had a monophasic disorder with complete resolution. Of the 29 patients (72.5%) with continuing symptoms, 15 (37.5%) have persistent static disease and 14 (35%) have a relapsing remitting course associated with further infections. All of the patients with tics fulfilled a diagnosis of PANDAS (Swedo SE et al., 1998). Although there was evidence of streptococcal infections during the majority of exacerbations, two patients had relapses after apparent viral infections, and one patient had an exacerbation after a routine vaccination. Nine of the 16 patients with motor tics (56%) fulfil a diagnosis of Tourette's syndrome ICD-10.

3.2.10. Clinical differences between tic and chorea subgroups

Comparisons between the clinical features are presented in table 3.b.

Table 3.b. Clinical comparison of tic and chorea subgroups. Psychiatric diagnoses according to ICD-10 criteria.

Characteristic	Tic (n=16)	Chorea (n=20)	p
Median age of presentation (range)	8 (2-13)	10 (1.5-13)	NS
Proportion male	69%	35%	0.04
Median length of follow-up (range)	2.5 (0.2-13)	0.45 (0.2-17)	0.001
Continuing movement disorder	94%	55%	0.01
Any psychiatric disorder	69%	50%	NS
Emotional disorder	50%	40%	NS
Obsessive-compulsive disorder	50%	10%	.02
Generalised anxiety	19%	25%	NS
Major depression	12.5%	20%	NS
Conduct disorders (all types)	25%	20%	NS
Hyperkinetic disorders (all types)	19%	15%	NS

As can be seen, chorea was more common in females than males. The converse was true in the tic subgroup. There were no other clear differences between the subgroups, although persistence of movement disorder and the presence of obsessive-compulsive disorder were more common in the tic subgroups. This however could at least partly be explained by the prolonged disease duration in the tic subgroup compared to the chorea subgroup.

3.3. Post-streptococcal acute disseminated encephalomyelitis (n=10)

3.3.1. Introduction

Although GAS classically causes CNS syndromes characterised by extrapyramidal movement and psychiatric disorders, it has been previously recognised that GAS can induce a broader spectrum of immune mediated CNS disorders, including disseminated inflammatory CNS disorders such as acute disseminated encephalomyelitis (ADEM) (Jorens PG et al., 2000). As part of my previous research into ADEM, I was able to recognise a number of cases with ADEM after GAS infection. The ADEM cases were notable because of the high incidence of basal ganglia lesions. In addition, the ADEM cases were clinically notable because of the high incidence of extrapyramidal movement and psychiatric disorders. I now present 10 cases of post-streptococcal ADEM seen at Great Ormond Street hospital between 1995 and 2000.

3.3.2. Demographics, past medical history and family history

There were eight males and two females, with a mean age of 6.8 years (range 3-14 years). None of the patients had any significant past medical history. One patient's father had Tourette's syndrome.

3.3.3. Precipitating infectious illness

All 10 patients had a preceding clinical pharyngitis before presentation. All had laboratory evidence of GAS infection. The average latency between pharyngitis and the onset of neurological symptoms was 11.5 days (range 4-18 days). Nine post-streptococcal ADEM (PSADEM) patients had elevated streptococcal titres in the acute phase, with a reduction in the convalescent period. In 1 patient the streptococcal serology was within the normal range, although serology performed 1 week later demonstrated a significantly elevated anti-DNase B level supporting recent infection. Serological ASOT was significantly elevated in the PSADEM patients (mean 424 IU/ml, SD 292, range 53-1060 IU/ml) compared to the neurological control group (mean 148 IU/ml, SD 139, range 53-655 IU/ml, p=0.002) but were not significantly different from the streptococcal infection control group (mean 562 IU/ml, SD 593, range 53-3210 IU/ml, p=0.1).

Similarly, anti-DNase B titre was also significantly elevated in the PSADEM patients (mean 1090 IU/ml, SD 1475, range 134 to 5100 IU/ml) compared to the neurological control group (mean 205 IU/ml, SD 238, range 68 to 1330 IU/ml, p<0.001) but not compared with the streptococcal infection group (mean 525 IU/ml, SD 346, range 76 to 1530 IU/ml, p=0.2).

3.3.4. Movement disorders

The clinical features of the 10 patients are presented in appendix 2 (chapter 13). In summary, an extrapyramidal movement disorder occurred in five patients; three had axial rigidity, four had limb rigidity (one with cogwheeling), two had dystonic posturing, two had rest tremor, one hemidystonia, and one paroxysmal hemidystonia. No patient had chorea or tics.

3.3.5. Behavioural alteration

Behavioural disturbance was common during presentation (n=7) and was characterised by emotional lability (n=3), inappropriate laughter (n=3), echolalia and pallilalia (n=1), attention deficit (n=1), separation anxiety (n=1) and confusion (n=3).

3.3.6. Other clinical features

Somnolence, stupor, and coma occurred in five patients. Three patients required ventilation because of the depth of coma. Pyramidal weakness was also common (n=6).

3.3.7. Clinical outcome

Nine children were treated with intravenous methylprednisolone for three days. Clinical improvement was often rapid. Eight children had a monophasic illness (ADEM), although two (patients 7 and 10) had relapses two and 13 months after initial presentation. Both relapses were triggered by pharyngitis (in patient 7, GAS serotype M77 was cultured by throat swab during the relapse). They have both subsequently been treated with penicillin prophylaxis in an attempt to minimise the occurrence of further relapses.

Eight patients have made a complete recovery. One child has developed an obsessive-compulsive disorder (patient 3) and one hemidystonia (patient 6). There have been no other reported episodes of acute neurological dysfunction in a mean follow-up of 1.9 years. No patients had clinical carditis during their acute illness (echocardiogram was performed in 4, and all were normal).

3.4 Post-streptococcal Parkinsonism

3.4.1. Introduction

The spectrum of post-streptococcal neuropsychiatric disorders has generally conformed to hyperkinetic and dyskinetic movement disorders, associated with psychiatric (mainly emotional) disorders. However, I was involved in the following two cases that raised the possibility that the spectrum of post-streptococcal movement disorders was broader than dyskinetic movements alone, but also included akinetic-rigid syndromes akin to Parkinsonism. Case one was seen by myself in London in 2000, case two was seen and reported in the literature by Israeli colleagues and myself in 2003.

3.4.2. Case histories

3.4.2.1. Case one

A 15 year old boy presented with an acute personality change 10 days after an upper respiratory tract infection. He became extremely anxious and worried about his safety. One week later he had an oculogyric crisis and developed upper limb resting tremor and bradykinesia. This was followed by extreme daytime somnolence, lethargy and intractable hiccough. On examination, he would fall asleep if not stimulated and yawned continuously. Pupillary responses were poorly reactive to light and accommodation. There was tongue tremor, a positive glabellar tap and slow speech. Limb examination revealed rigidity with cogwheeling, bradykinesia and freezing. He had a stooped gait with poor arm swing. Positive results included an elevated ASOT (350 IU/ml), and a mirrored pattern of oligoclonal bands in both CSF and serum. CSF PCR for neurotropic viruses was negative. MRI brain showed enhancement of the

basal ganglia seen on T2-weighted imaging. He was treated with 50mg of levodopa twice a day (with carbidopa), which improved his sleep disorder and Parkinsonian signs, although he complained of insomnia. His abnormal clinical signs remained for 2 months following which the levodopa was withdrawn. He has made a complete recovery with no neurological or psychiatric sequelae at one year follow-up. In summary, this patient presented with a post-infectious (streptococcal) Parkinsonian syndrome with psychiatric and brainstem features.

3.4.2.2. Case 2

A 10-year-old girl was referred for evaluation of chorea five weeks after having had a sore throat for which she had not received antibiotic treatment. During the five week interim, she developed small, rapid, involuntary finger movements described as "piano playing" and her behaviour was quieter and more introverted than usual. She also manifested a stereotypy during which she would repeatedly stop what she was doing, stand on one foot "like a stork" and freeze in this position for several moments. Her physician diagnosed the former irregular hyperkinetic movements as chorea and he prescribed penicillin treatment. Neuroleptics were not recommended and the child was referred for neurological assessment.

The patient is the sixth of eight children of unrelated Ashkenazi parents. Her perinatal and developmental histories were normal; she had enjoyed good health and was not on any medications. There had been no other febrile illnesses, medical or emotional problems in the months prior to referral. Her maternal uncle suffered from rheumatic heart disease. There was no familial history of a psychiatric or movement disorder (e.g. Parkinson's disease, dystonia or Huntington's disease) and no neuroleptics or psychotropic medications were found during a house visit.

On examination, five weeks after the onset of the hyperkinetic movement disorder, her chorea and "stork posture" had resolved. However, signs of hypomimia were strikingly apparent. She had a "poker face", lips slightly apart without facial mimicry. Bradykinesia and increased limbs muscle tone were more noticeable on her left side with cogwheel rigidity. Her posture was stooped; hands and arms slightly flexed. Her gait was remarkable for absence of arm swing and presence of foot shuffling. She had no chorea, tremor, dystonia or cerebellar signs. Otherwise her physical examination was within normal limits. We evaluated the movement disorders with the motor section of the Unified Parkinson's Disease Rating Scale (UPDRS). Her movements were similar to those of Parkinson's disease and her total motor score was 28 [normal value 0] (Table 1).

Initial blood tests, including complete blood count, sedimentation rate, serum electrolytes, liver and kidney functions, were all within normal limits. Throat swab at the time of examination was positive for streptococci-non-group-A. Anti-streptolysin O titer was elevated, 585 IU/ml [normal < 200 IU/ml]. Further laboratory evaluation demonstrated normal level of thyroid stimulating hormone, anti-nuclear antibody, C3, C4, copper and caeruloplasmin. Brain MRI including basal ganglia imaging, EEG and echocardiogram were normal. DYT1 mutation for torsion dystonia was negative. Neurological examinations three and six months after her initial referral revealed gradual fading of the parkinsonian signs with residual movement abnormalities in gait and posture at three months follow-up (figure 1). The total UPDRS preformed in her follow up visits after three and six months was 18 and 15 respectively (Table 3.c).

Table 3.c. Motor UPDRS (Unified Parkinson's Disease Rating Scale) evaluation on presentation and follow up

#	Motor Examination	At presentation	3 months follow up	6 months follow up
18	Speech	1	1	1
19	Facial expression	3	1	1
20	Tremor at rest	0	0	0
21	Action or postural tremor- Right hand	0	0	0
	- Left hand	0	0	0
22	Rigidity - Right arm	3	0	0
	-Left arm	3	2	1
	-Right leg	2	0	0
	-Left leg	2	1	0
23	Finger taps -Right	1	1	0
	-Left	1	1	1
24	Hand movement -Right	2	2	2
	-Left	2	2	2
25	Rapid alternating movements -Right hand	1	1	1
	-Left hand	1	2	2
26	Leg agility -Right	1	0	0
	-Left	1	1	1
27	Rising from chair	0	0	0
28	Posture	1	0	0
29	Gait	1	1	1
30	Postural stability	1	1	1
31	Body bradykinesia and hypokinesia	1	1	1
	Total score	28	18	15

Each task is graded on a scale from 0-4:

0= normal; 4= severe impairment, can barely perform the task.

3.4.3. Hypotheses on post-encephalitic Parkinsonism

These descriptions of post-infectious Parkinsonism (both cases after streptococcal infection) suggest that the spectrum of post-streptococcal movement disorders is broader than previously described and includes an akinetic-rigid syndrome. The cases (particularly case one) were reminiscent of encephalitis lethargica, an infection-associated syndrome of encephalitis, Parkinsonism, psychiatric disturbance, sleep disturbance and brainstem features. The novel finding that streptococcus may induce an immune mediated Parkinsonian syndrome required further study, and patient ascertainment.

3.5. Encephalitis lethargica (n=20)

3.5.1. Patient recruitment of encephalitis lethargica

In view of the two cases described in the previous section, I recruited further patients presenting with a similar syndrome of acute onset, post-infectious or immune mediated Parkinsonism. This became possible after I presented the index cases at regional and national conferences. Cases were subsequently referred to Great Ormond Street Hospital or the National Hospital for Neurology and Neurosurgery for further assessment.

All included patients were referred between April 1999 and May 2002 with a new onset CNS dysfunction resulting in an encephalitis lethargica-like syndrome (sleep disorder and associated lethargy, Parkinsonism and neuropsychiatric disorders).

3.5.2. Demographics and past medical history

The demographics and clinical characteristics of the patients with the EL phenotype are presented in appendix 3 (chapter 13). Other than 'high functioning' autism (n=1) and Down's syndrome (n=1), there was no past medical history of note.

3.5.3. Preceding infectious illness and aetiological investigations

Eleven patients (55%) had an infection shortly before disease onset. The infections were described as upper respiratory tract infection (n=6) and tonsillitis (n=5). Patient presentation was described as acute, subacute or insidious in 35%, 50% and 15% respectively. Investigations were tailored according the age of the patient and presentation characteristics. Negative investigations are presented in appendix 4 (chapter 13).

Two patients had concomitant tonsillitis at assessment; Group A streptococcus was grown from the throat in both patients. ASO titres were performed in all patients and controls. The data is presented in table 3.d. The mean ASOT in the EL-like cohort was significantly elevated compared to the child dystonia ($p<0.005$), adult neurology ($p<0.0001$) and adult healthy controls ($p<0.0001$). ASOT was elevated in 65% of EL patients, but normal in 35%. There was no statistical difference between the EL group and the child streptococcal group ($p= 0.17$)

Table 3.d. Streptococcal serology in encephalitis lethargica (EL) cohort compared to control groups

Group	EL	Child streptococcus controls	Child dystonia controls	Adult neurology controls	Adult healthy controls
ASOT IU/ml mean (95% confidence intervals)	301 (198-404)	332 (269-395)	151 (99-203)	133 (98-168)	122 (101-143)

3.5.4. Movement disorders

3.5.4.1. Parkinsonism

All patients had signs consistent with Parkinsonism. Twelve of 20 patients had a Parkinson's syndrome according to UKPDS Brain bank criteria; six had all three cardinal features (bradykinesia, rigidity and rest tremor), two had bradykinesia with postural instability, two had bradykinesia with rigidity, and two bradykinesia with rest tremor. The other eight patients had isolated features of the Parkinsonian phenotype; bradykinesia / akinesia (n=6), rest tremor (n=1) and rigidity with rest tremor (n=1).

3.5.4.2. Dyskinesias

In addition to Parkinsonism, 11 patients had evidence of concomitant dyskinetic movement disorders; dystonia (n=6) of whom 5 had a generalized dystonia, chorea/hemiballismus (n=2), motor tics (n=2), stereotypies (n=2), facial grimacing and blepharospasm (n=1 each). Three patients had oculogyric crises.

3.5.5. Behavioural alteration and psychiatric disorders

The psychiatric manifestations are described in appendix 3 (chapter 13). Seventeen of 20 patients had psychiatric disturbance. Mutism occurred in 10 patients. Emotional disorders were also common and included depression DSM-IV (n=6), obsessive-compulsive disorder DSM-IV (n=3) and anxiety (n=2). Apathy and catatonia occurred in four and three patients respectively.

3.5.6. Other clinical features

3.5.6.1. Sleep disturbance and lethargy

19 of 20 (95%) patients had sleep disturbance; hypersomnolence (n=12), insomnia (n=2) and sleep inversion (n=5). It was usually possible to rouse the somnolent patients without difficulty, but they would fall asleep if not stimulated. Other sleep abnormalities included vivid nightmares (n=2) and sleep walking (n=1). 10 patients had lethargy, which was in excess of what would be expected for the degree of motor weakness.

3.5.6.2. Other features

Five patients had profound reduction in consciousness and required ventilation. Eight patients had ocular abnormalities (4 ophthalmoplegia, 3 pupillary disturbance, 1 ptosis, 1 optic neuritis). Hyperventilation and nocturnal bradycardia were also observed (appendix 3). Seizures and memory loss occurred in 3 patients each. Symptoms of intracranial pathology were common, including headache (n=6), photophobia (n=3) and meningism (n=2). Other features included incontinence (n=3) and limb pains (n=2).

3.5.7 Clinical outcome

10 patients had a monophasic illness, seven a relapsing polyphasic course, two static disease and one had progressive disease until death. After a mean follow-up of five months (range 2 months-14 months), only five patients have made a complete recovery to date; Of the 15 patients with continuing impairments, six have a persisting

movement disorder and ten have disabling neuropsychiatric disturbance (one patient has both movement and psychiatric disorders).

3.5.8. Histopathology of encephalitis lethargica

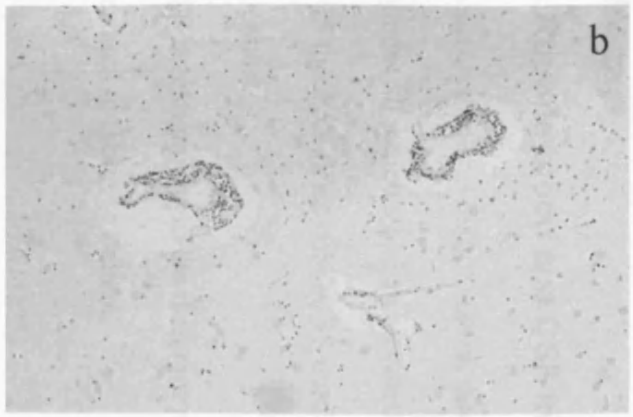
One patient died of the acute encephalitis lethargica illness. The case and histopathological findings are now presented.

3.5.8.1. Case history

A 69 year old male with a history of chronic obstructive airways disease presented following a febrile illness with profound somnolence and confusion. He became withdrawn, apathetic and stopped caring for himself. He subsequently developed bradykinesia, bilateral rigidity with cogwheeling. There were no pyramidal, cerebellar or sensory signs. MR neuroimaging revealed bilateral enhancement of the basal ganglia with associated swelling of the striatum and globus pallidus. CSF revealed no cells but intrathecal oligoclonal bands. CSF pcr for Herpes simplex virus, varicella zoster virus and human herpes virus 6 and 7 were negative. His neurological syndrome progressed over 3 weeks and was complicated by a lower respiratory tract infection, subsequent respiratory failure and the patient died.

3.5.8.2. Histopathological findings

Histological examination revealed perivascular lymphocytic cuffing that was best developed in the basal ganglia, but also seen to a lesser extent in the cerebral cortex and cerebellum (Figure 3.6a). Changes were most extensive in the putamen, globus pallidus and the amygdala. There was no neuronophagia and no viral inclusions. Staining of striatal sections identified the infiltrating cell populations as T and B



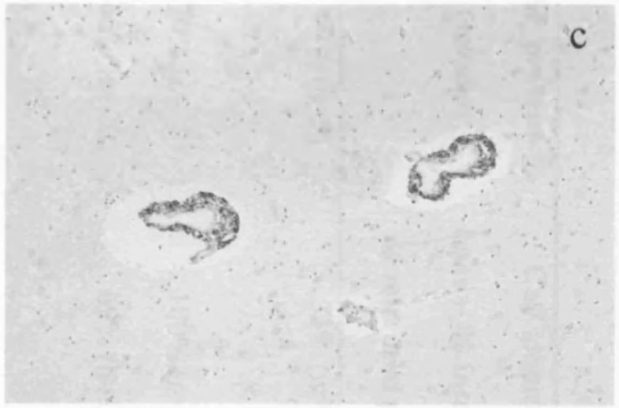


Figure 3.6.a. Perivascular cuffing

Figure 3.6.b. T-lymphocyte staining.

Figure 3.6.c. B-lymphocyte staining.

lymphocytes (Figure 3.6b+c). Staining for amyloid and complement was negative. There were reactive astrocytes and activated macrophages in the striatal parenchyma.

3.6. Investigation of post-streptococcal neuropsychiatric syndromes

3.6.1. Routine blood and CSF investigations

The mean erythrocyte sedimentation rate (ESR) was elevated in all phenotypes, although not all patients had an elevated ESR (>20 mm/hr). The mean ESR was 26.5 mm/hr in the dyskinesia cohort, 38 mm/hr in the PSADEM cohort, and 30 mm/hr in the EL cohort.

The CSF investigations in the different post-streptococcal neuropsychiatric syndromes are presented in table 3.e below.

Table 3.e. CSF findings in the different post-streptococcal neuropsychiatric syndromes (number tested in brackets).

Syndrome	**CSF white cell count- % elevated** *	**CSF protein- % elevated** **	**CSF oligoclonal bands- % intrathecal or mirrored pattern**
Dyskinesias (n=40)	11% (n=9)	67% (n=9)	33% (n=9)
PSADEM (n=10)	40% (n=10)	70% (n=10)	100% (n=3)
Encephalitis lethargica (n=20)	12% (n=16)	60% (n=20)	69% (n=13)

*All CSF white cells were lymphocytes

** CSF protein elevated over 0.4 g/l

As can be seen, there is non-specific evidence of inflammation and immune activation in one third of the main dyskinesia group and the majority of EL and ADEM patients. There is frequently evidence of mirrored IgG clones in the CSF and serum (consistent with a systemic clonal expansion of IgG with cross over into the CSF). Occasionally, mainly in the EL phenotype, there is intrathecal synthesis of IgG clones. However, sometimes there is no evidence of IgG clones in CSF or serum using isoelectric focussing. However, it should be noted that the sample timings were not all during the acute phase.

3.6.2. Neuroimaging

Magnetic resonance neuroimaging is an important part of any neurological syndrome investigation. MR neuroimaging is often done to exclude important aetiological causes such as space occupying lesions. Most of the post-streptococcal neuropsychiatric patients underwent MR neuroimaging, and the results are reported here. Clearly, the MR neuroimaging is very important in the classification of some of the syndromes such as acute disseminated encephalomyelitis where disseminated inflammatory CNS lesions are characteristic and required for diagnosis. The MR neuroimaging results are summarised in table 3.f below.

Table 3.f. Abnormal imaging (inflammatory lesions) in the different clinical phenotypes, including presence of basal ganglia lesions.

Syndrome (patients with MR neuroimaging)	Percentage abnormal	Percentage with basal ganglia inflammatory changes	Percentage with inflammatory changes elsewhere in CNS
Dyskinesia (n=26)	12%	12%	0%
PSADEM (n=10)	100%	80%	70%
EL (n=20)	40%	40%	10%

As can be seen, in the dyskinesia cohort, the MR imaging was usually normal. By contrast, in PSADEM and EL, imaging was often abnormal and is described in detail:

3.6.3.1. Post-streptococcal acute disseminated encephalomyelitis

The inflammatory lesions were specifically localised to the following sub-regions in the PSADEM patients: Basal ganglia lesions were identified in 8 of 10 patients (80%). This involved the caudate (50%), putamen (60%), and globus pallidus (40%). Other deep grey structures involved included the thalamus (60%), subthalamus (30%), and substantia nigra (50%). Lesions were also seen in the supratentorial white matter (70%), brainstem (40%), cerebellum (20%), and spinal cord (10%). Only 2 patients had no reported basal ganglia lesions, although one of these had a thalamic lesion. In comparison, basal ganglia lesions were reported in only 18% (4/22) of control ADEM (non-streptococcal) patients during the same time period (Dale et al., 2001). Using Fisher's exact test, MRI basal ganglia lesions were statistically more prevalent in the PSADEM group compared to non-streptococcal ADEM (p=0.0002). All lesions were

T2 hyperintense, whereas T1 sequences were normal. Gadolinium was not routinely given. Examples of the MR neuroimaging are given in figures 3.7 and 3.8.

Figure 3.7. MR neuroimaging of PSADEM patient. Brain T2-weighted (coronal section) demonstrating lesions involving the left caudate (short arrow) and bilateral putamen nuclei (long arrow).

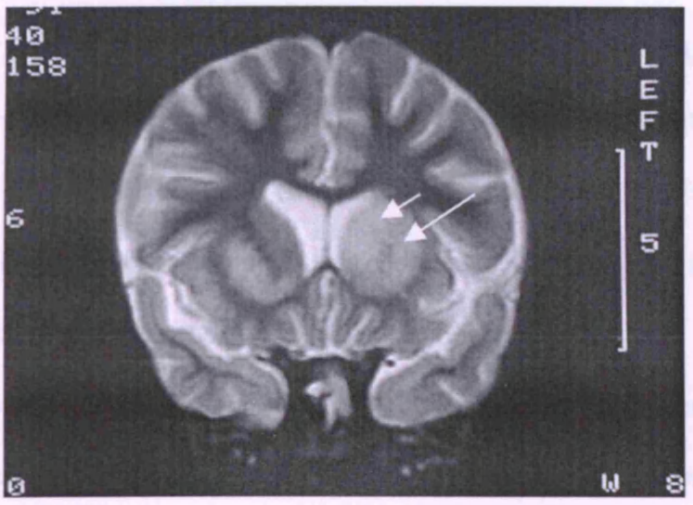
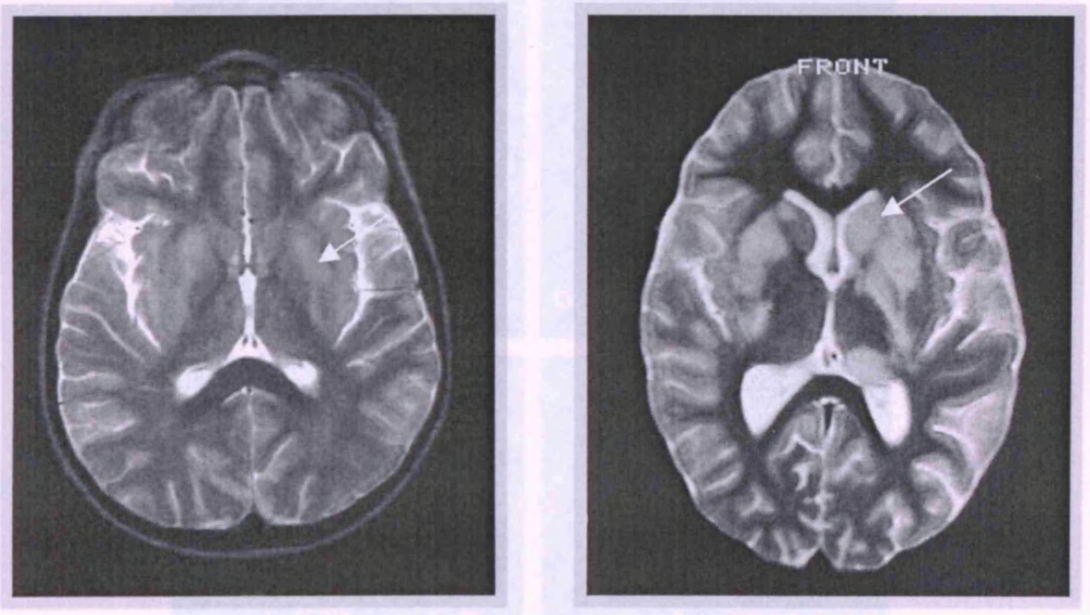


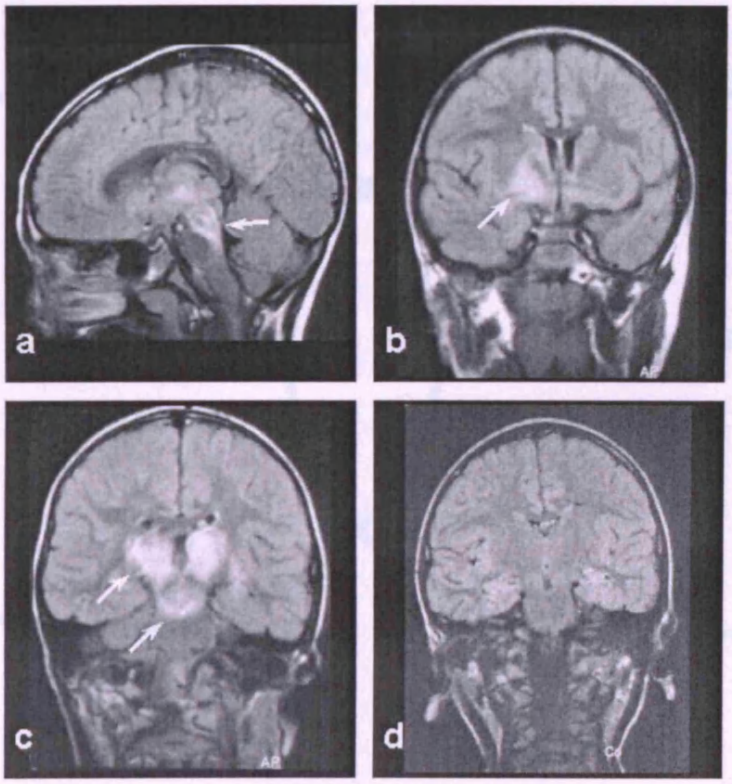
Figure 3.8. MR neuroimaging of two separate PSADEM patients showing predominant basal ganglia lesions in both patients (arrows).



3.6.3.2. Encephalitis lethargica

Magnetic resonance neuroimaging was performed in all patients and was abnormal in 8 (40%). All abnormal images had high signal lesions on T2 imaging. The abnormalities were present in basal ganglia (n=8), midbrain/tegmentum (n=5), thalamus (n=2), cerebral peduncle (n=1) and temporal lobe (n=1) (Figure 3.9). Three patients had convalescent imaging during the recovery phase of the illness, which showed resolution of the abnormalities (Figure 3.9).

Figure 3.9. MRI brain in a somnolent EL patient, showing inflammatory lesions in the midbrain and periaqueductal grey matter (Figure 3.9a), right putamen (Figure 3.9b) and bilateral thalami and midbrain (Figure 3.9c). Convalescent imaging showing resolution of the inflammatory changes in the thalami and midbrain (Figure 3.9d).



3.7 Summary of proposed post-streptococcal neuropsychiatric disorders

This series is not epidemiological in design, but is a selected patient population. Also the patients were recruited due to the presence of extrapyramidal movement disorders. For these reasons, this description cannot truly represent the true spectrum of post-streptococcal neuropsychiatric disorders in the childhood population. However, this data has highlighted new and important post-streptococcal neuropsychiatric phenotypes. The findings of the clinical part of this thesis can be summarised by the following simple (Figure 3.10) and detailed (Figure 3.11) figures:

Figure 3.10. Summary of the common clinical features of post-streptococcal neuropsychiatric syndromes.

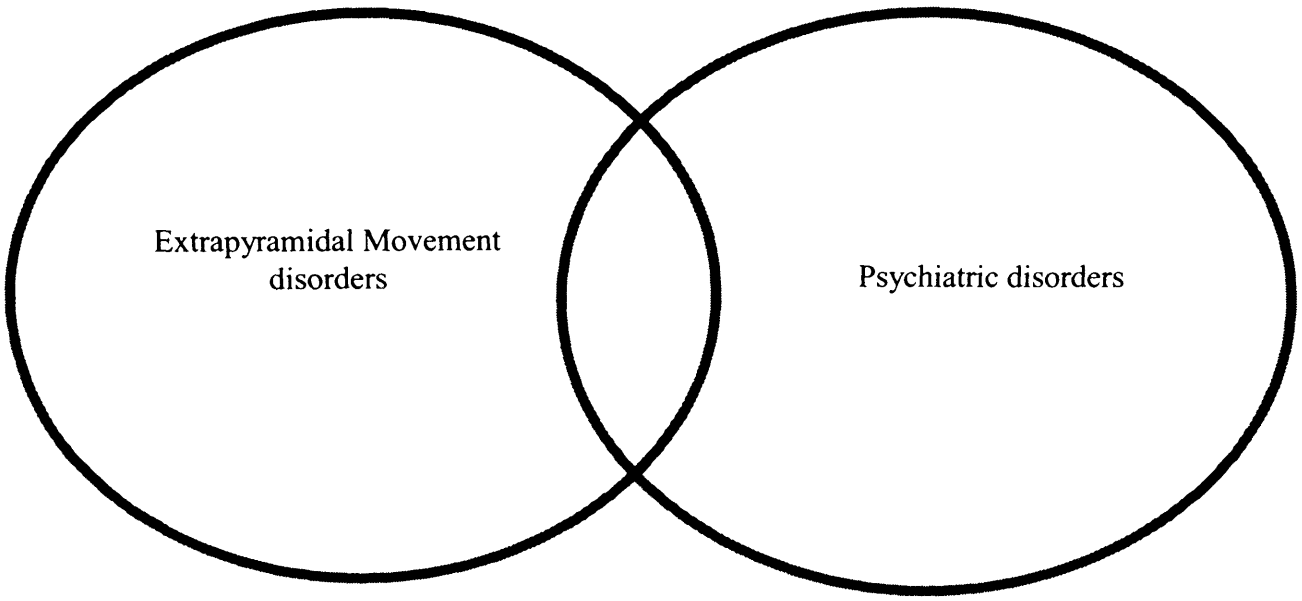
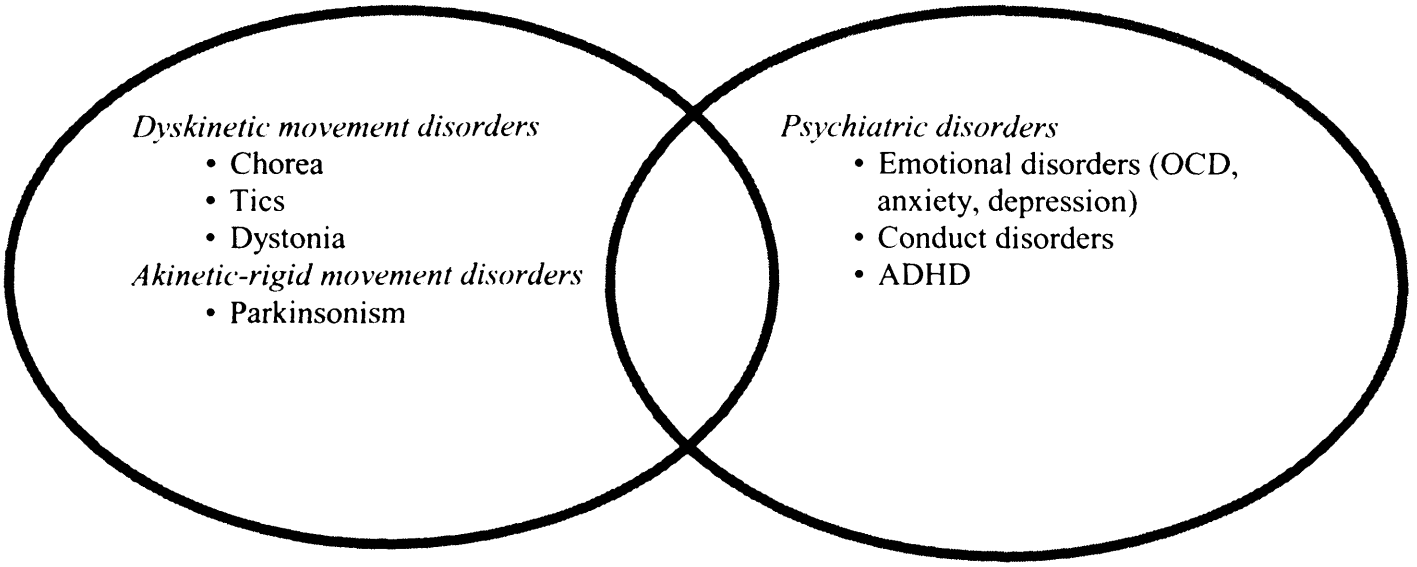


Figure 3.11. Detailed summary of the clinical features of post-streptococcal neuropsychiatric syndromes.



The clinical features can also be summarised according to the clinical syndrome (Table 3.g):

Table 3.g. Summary of proposed post-streptococcal neuropsychiatric phenotypes

Phenotype	Movement disorder	Psychiatry	Other features	Neuroimaging
SC	Chorea	Emotional disorders plus	Insomnia	Normal
PANDAS	Tics	Emotional disorders plus	-	Normal
PSADEM	Dystonia-Parkinsonism	Emotional disorders plus	Encephalopathy, pyramidal signs	Basal ganglia and other CNS lesions
EL	Parkinsonism plus	Emotional disorders	Somnolence, lethargy	Basal ganglia leisons

In addition, the post-streptococcal neuropsychiatric phenotype includes the presence of sleep disorders. Notably, in the dyskinetic phenotype, the patients were more likely to suffer from insomnia or reduced sleep. In contrast, the Parkinsonian patients were more likely to suffer from somnolence or increased sleep.

3.8. Anti-neuronal antibody findings in the post-streptococcal neuropsychiatric syndromes

The thesis by Andrew J Church (findings to be discussed in chapter 4) examined the prevalence of anti-neuronal antibodies (against basal ganglia antigens) in the patients with post-streptococcal neuropsychiatric syndromes. A summary of the anti-neuronal antibody results is presented in Table 3.h.

As can be seen, there were common autoantibodies in the serum of patients with post-streptococcal dyskinesia, ADEM and Parkinsonism. These findings have been published (Church et al., 2002; Dale et al., 2004). The autoantibodies bound to autoantigens of molecular weight 40, 45 and 60 kDa. In addition, in the encephalitis lethargica group, a 98 kDa autoantigen was recognised more frequently in patients than controls. Identifying these autoantigens will be the immunological aim of this thesis.

Table 3.h Anti-basal ganglia antibodies (ABGA) in post-streptococcal neuropsychiatric syndromes and controls as measured by Andrew J Church (Church et al., 2004; Dale RC et al., 2004; Dale RC et al., 2001). There were other bands seen in some patients or controls, thought to be of lesser significance and not reported in this table.

Group	Percentage with positive ABGA (Western blotting)	Autoantigen (kDa) binding (percentage)			Reference
		40	45	60	
Post-streptococcal dyskinesia (n=40)	92.5%	47.5%	40%	42.5%	Church AJ et al., 2004
Post-streptococcal ADEM (n=10)	100%	10%	0%	80%	Dale RC et al., 2001
Encephalitis lethargica (n=20)	95%	55%	30%	55%	Dale RC et al., 2004
Child neurology controls (n=100)	4%	1%	0%	1%	Church AJ et al., 2004
Child streptococcal controls (n=40)	2%	0%	0%	2%	Church AJ et al., 2004
Child autoimmune controls (n=50)	10%	4%	0%	2%	Church AJ et al., 2004
Adult neurology controls (n=50)	2%	2%	0%	0%	Dale RC et al., 2004
Adult healthy controls (n=50)	4%	0%	0%	0%	Dale RC et al., 2004

Chapter 4. Introduction- immunopathogenesis of post-streptococcal neuropsychiatric disease

4.1. Concepts of CNS autoimmunity

4.1.1. Blood brain barrier

4.1.1.1. Composition and integrity

The blood brain barrier (BBB) protects the central nervous system from fluctuations in serum constituents and strongly limits the access of cells (such as immune cells) to the brain. The BBB consists of the cerebrovascular endothelium, the choroid-plexus epithelium and the arachnoid membrane. There are three properties that distinguish the brain capillary endothelial cells from their peripheral counterparts, namely:

1. Dedicated tight junctions that are fusion sites between cells on adjacent plasma membranes.
2. Low rates of endocytosis.
3. Specific transport and carrier molecules (Gloor SM et al., 2001). Although these characteristics make the BBB a robust barrier from the rest of the body, proteins and cells are still capable of accessing the brain either by diffusion through pores or via active transcellular passage (discussed later). Consequently under physiological conditions, CSF protein concentrations are normally 0.5% or less of the respective plasma or serum concentration. The proteins diffuse across the BBB according to their size (or radius) with smaller proteins diffusing more rapidly. The degree of diffusion obviously alters (increases) when the BBB is damaged. There are other features of the BBB that protect the brain from immune cell access:

a) Under physiological conditions, the endothelial cells of the BBB express very low levels of adhesion molecules (required for leucocyte migration) (Bart J, 2000).

b) Transforming growth factor- (TGF-β) is made in the CNS and is present in the CSF. TGF-β suppresses T-lymphocyte proliferation, down-regulates adhesion molecules and markedly diminishes leucocyte migration (in contrast to Interleukin-1, Interferon-γ and lipopolysaccharides). TGF-β therefore minimises immunological surveillance of the brain.

However there are a number of recent developments regarding the brain and immune surveillance that require specific discussion. It used to be thought that there was no antigen drainage from the brain. However, it has recently been shown that CSF and brain fluid move through the brain via Virchow-Robin spaces to lymphatic-like systems beneath the cribriform plate, and subsequently along cranial and spinal nerve roots (Kida S et al., 1995). Antigen from the brain can then flow into the cervical lymph node system and therefore be seen by the immune system (Cserr HF, Knopf PM, 1992). These CNS-derived antigens can provoke a Th2 lymphocyte and B lymphocyte response (Harling-Berg CJ et al., 1999).

In conclusion, although the BBB provides the brain with an important barrier, it is by no means impenetrable. Indeed proteins gain access into the CNS at a rate dependent upon the protein size. Likewise, immune surveillance does occur in the brain, although at a very low level, and brain antigens can drain into the cervical lymph system resulting in potential immune activation.

4.1.1.2. Blood brain barrier and T-lymphocytes

There is no doubt that T-lymphocytes are capable of surveilling the CNS parenchyma, although this occurs at a low level (Hickey WF, 1999). If there is strong

immunological activity in the body, irrespective of whether the CNS is involved, there will be an elevated number of T-cells in the CNS (Hickey WF and Kimura H, 1987). What is clear is that only activated T-cells, particularly secondary to recent antigen stimulation (Fritz RB et al., 2000) are capable of gaining entry into the CNS. Resting or naïve T-lymphocytes cannot gain access (although this has been partly questioned recently). The level of surveillance is partly related to the low number of adhesion molecules on the brain endothelial membranes, and also to the lower level T-lymphocyte migration into the cerebrum compared to the spinal cord (Phillips LM and Lampson LA, 1999).

It appears that there are no homing mechanisms for T-cells to find a CNS antigen. Rather the T-cells perform 'antigen-seeking' in a random manner (Carrithers MD et al., 2000). If the T-cells do encounter their CNS antigen, they accumulate there and cause secondary inflammation (Hickey WF, 1991). Despite the fact that T-cells can gain access to the CNS, they do find the CNS environment hostile and rapidly die via an apoptotic mechanism (Bauer J et al., 1998). Another interesting fact is that not all activated T-cells may have a detrimental effect on the CNS. Indeed some T-cells have a protective effect and via T-cell produced neurotrophic factors can result in neuronal repair (Hohlfeld R, 2000).

4.1.1.3. Blood brain barrier and antibodies or B-lymphocytes

Antibodies can gain access to the CNS in a number of ways. Firstly under physiological conditions, antibodies (like other proteins) diffuse via pores into the CSF and CNS, albeit at low levels (0.1-0.4%) of serum levels (Eeg-Olofsson O et al., 1981). Clearly, if the BBB is disrupted then antibodies could diffuse more readily into the CNS. Practical markers of demonstrating antibody presence in the CSF include performing isoelectric focussing electrophoresis of CSF and serum. This can

demonstrate clones of IgG in the respective compartments and can differentiate whether antibodies are in the CSF alone (intrathecal production) or are also represented in the serum (mirrored pattern).

Alternatively, antibodies can be synthesised locally in the CNS by B-lymphocytes that have entered the CNS, clonally expanded and differentiated into antibody secreting plasma cells. A recent study demonstrated that B-lymphocytes, after activation, are capable of traversing the intact BBB, locating their specific antigen within the brain, and differentiating into antibody producing plasma cells (Knopf PM et al., 1998). It would therefore appear that B-lymphocytes survey the normal CNS as do T-lymphocytes. It is not clear whether this process is dependent upon T-cell help.

4.1.2. Molecular mimicry

4.1.2.1. Concepts of molecular mimicry

There are a large number of ways in which beta-haemolytic Streptococci can induce immune mediated neuronal damage, including polyclonal activation of lymphocytes, superantigen activation, cytokine activation or exposure of previously unexposed (cryptic) self-antigens. A further potential mechanism is termed molecular mimicry. The molecular mimicry hypothesis states that a susceptible host encounters a micro-organism that has antigens that are immunologically similar to the host antigens, but differ enough to induce an immune response in the host (Albert LJ and Inman RD, 1999). This hypothesis has remained popular as a mechanism for autoimmune induction despite the paucity of evidence that is clinically relevant in humans.

4.1.2.2. The host and molecular mimicry

Although the molecular mimicry hypothesis is appealing, there are a number of important issues that have been recognised over the last decade.

The immune system recognises itself and has developed mechanisms to avoid attacking itself (self tolerance). Indeed, self-reactive T-cells are negatively selected in the thymus and deleted by apoptosis, or rendered anergic (Ohashi PS et al., 1996; Goodnow CC et al., 1996). However, some T-cells are not deleted, sometimes because the autoantigens are present peripherally at a very low level (or cryptic, i.e. unseen) (Sercarz EE et al., 1993). Also high avidity T-cells are deleted in the thymus, whereas low affinity T-cells may escape deletion and may be subsequently capable of inducing autoimmune disease (Fourneau JM et al., 2004). Loss of tolerance may depend on a number of sequences such as presentation of the antigen (e.g. by an appropriate antigen presenting cell and major histocompatibility complex) and also by a secondary signal such as cytokine secretion (Rose NR, 1998). Similarly, autoantibodies are also present in healthy individuals. In one study, 3% of monoclonal antibodies reactive to different virus proteins cross-reacted with normal tissue antigens in mice (Srinivasappa J et al., 1986). In other words, autoantibodies themselves are not uncommon although are usually of low levels, low avidity and of little pathogenic relevance.

4.1.2.3. Immunogenic epitopes: sequence versus structural homology

In the early conception of molecular mimicry, it was proposed that amino acid sequences were required to be identical between the host and the organism to activate T-cell receptors or bind cross-reactive auto-antibodies. Early protein sequence database comparisons stipulated that a seven amino acid sequence or more was required to be theoretically significant (Karlsen AE and Dyrberg T, 1998). However it soon became apparent that immune reactivity (particularly by T-cells) was degenerate and did not require such specific interaction. Indeed only a few crucial residues (amino acids) were required to result in binding of T-cell receptors (Kohm AP et al.,

2003). Subsequently it has become clear that the structure or conformation of antigens is also highly relevant, and this homology may result in proliferation of T or B cells (Kohm AP et al., 2003).

4.1.2.4. Problems in demonstrating molecular mimicry in clinical disease.

Autoimmune diseases have been linked to infectious diseases for decades. One problem with proving a link is to demonstrate a reliable temporal association between a particular organism and disease. This is difficult because the infection may have long resolved before the disease is clinically evident (Albert LJ and Inman RD, 1999). Secondly, molecular mimicry may be a secondary phenomenon caused by an alteration in host antigens (revealing previously cryptic antigens) and the creation or alteration of neoepitopes. Therefore reactive T-cell or autoantibodies could be formed as a consequence of, rather than the cause of, tissue injury.

In summary, although the molecular mimicry hypothesis is tempting, there are a number of critical problems that must be recognised or overcome:

1. Antigenic mimicry alone may not overcome self-tolerance, and may require co-stimulation such as cytokines.
2. Antigenic homology or proliferation does not by itself indicate pathogenic mimicry.
3. A tissue-specific antibody response does not, by itself, indicate pathogenic mimicry, but may be secondary to tissue injury.
4. Antigen mimicry may elicit tolerance of the host immune response, rather than autoimmunity.
5. A genetic vulnerability is likely to be relevant in most autoimmune diseases in addition to the environmental trigger. Most genetic vulnerability is related to antigen presentation and major histocompatibility complexes (MHC).

4.1.2.5. Examples of molecular mimicry in humans

A number of microorganisms have been incriminated in putative autoimmune diseases as described in table 4.a.

Table 4.a. Proposed molecular mimicry in human diseases (Albert LJ and Inman RD, 1999)

Autoimmune disease	Proposed antigen	Proposed pathogen	Immune cross-reactivity
Type 1 Diabetes mellitus	GAD 65	Coxsackievirus	T cell
Rheumatoid arthritis	HLA-DRB1, Heat shock protein 60	Mycobacterium tuberculosis, heat shock protein 65	T and B cells
Multiple sclerosis	Myelin basic protein	Multiple viruses	T cells
Spondyloarthropathies	HLA-B27	Multiple gram negative bacteria	B cells
Graves disease	Thyrotropin receptors	Yersinia enterocolitica	B cell

Although there are a number of lines of evidence supporting the role of molecular mimicry in these diseases, the evidence is incomplete and remains controversial (Albert LJ and Inman RD, 1999). Perhaps the best evidence, and pleasingly for the sake of this thesis, comes from work in rheumatic fever (RhF). RhF remains the prototype of post-infectious immune mediated disorders. The primary advantage in RhF investigation is that beta-haemolytic Streptococci have been conclusively proven

to be the cause of RhF. Most of the molecular mimicry data has focussed on the rheumatic heart disease (the immunological data related to the brain will be presented shortly). RhF patients have antibodies that bind to the M protein of Type 5 streptococci that cross-react with myocardial tissue (Zabriskie JB et al., 1966; Dale JB and Beachey EH, 1985; Kaplan MH and Dallenbach FD, 1961) and can result in the destruction of rat heart cells in vitro (Adderson EE et al., 1998). Further studies have demonstrated that infiltrating T-lymphocytes from RhF heart lesions are cross-reactive with immunodominant epitopes of the streptococcal M protein (Guilherme L et al., 1995). This group have specifically shown CD4+ T-cell cross-reactivity between the M5 protein and human mitral valve proteins and myosin peptides (Guilherme L and Kalil J, 2002).

4.1.3. Autoantibody criteria for pathogenicity

The presence of antibodies in serum does not necessarily infer pathogenicity. For example, antibodies could be produced as part of tissue damage. In order to demonstrate that a disorder is antibody mediated autoimmunity, five criteria must be fulfilled (Archelos JJ and Hartung HP, 2000);

1. Presence of auto-antibodies.
2. Presence of antibodies in target tissue.
3. Induction of disease in animal model by passive transfer of antibody.
4. Induction of disease in animal model by auto-antigen immunisation.
5. Improvement of clinical symptoms after removal of antibodies with plasma exchange.

A number of proposed autoimmune CNS disorders are summarised in table 4.b., including the evidence acquired to date in each condition (Lang B et al., 2003).

Table 4.b. Criteria for autoantibody pathogenicity in proposed immune mediated CNS disorders

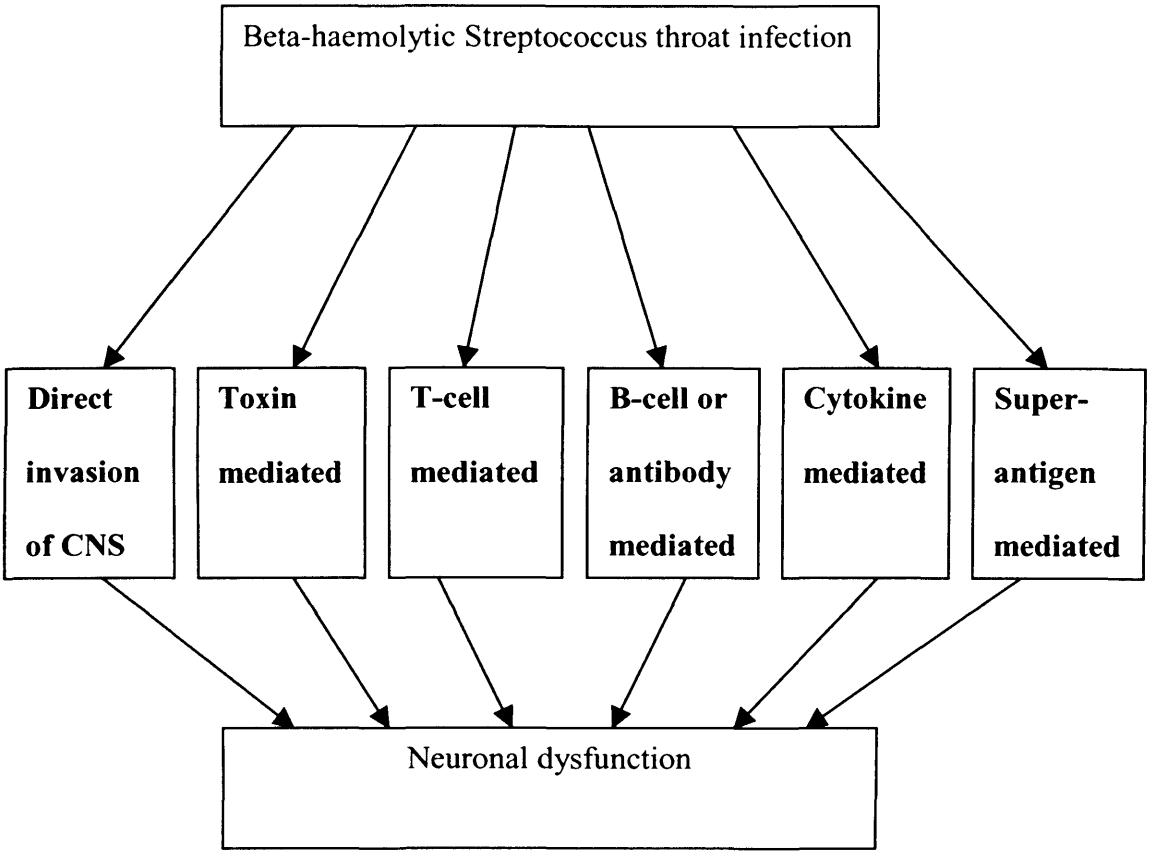
Antibody specificity	Disorder	Ab's in CSF	Response to immunotherapy	Pathogenicity of autoantibodies	Associated condition
Anti-Hu, Yo, Ma1/2, Cv, Tr	Paraneoplastic Neurological syndromes	+*	Little	None	Multiple tumours
Anti-VGCC	Paraneoplastic cerebellar degeneration	+*	Some	Anti-VGCC antibodies from LEMS down-regulate VGCC	SCLC
Anti-VGKC	Morvan's syndrome	?	Yes	Anti-VGKC antibodies in NMT patients suppress K^+ currents in human neuroblastoma cells	thymoma + NMT
	Reversible Limbic encephalitis	?	Yes		thymoma
Anti-GluR3	Rasmussen's Encephalitis	?	Transient effects in some patients	Antibodies produced to GluR3 proteins elicit RE symptoms	Nil
Anti-GAD	Stiff-Man Syndrome	+*	Good response		-
	Progressive Cerebellar ataxia	+		Antibodies inhibit GABA-mediated transmission in Purkinje cells	-
	Batten's disease	?	NK		Deletion in CLN3 gene
Anti-DNA	Neuropsychiatric SLE	+	Yes	Antibodies mediate apoptopic death of neurones	SLE
Anti-phopholipid			Response to steroids		
Anti-basal ganglia antibodies	Post-streptococcal CNS syndromes	+	Some	Antibodies mediate repetitive behaviours in rats	Group A Streptococcal infection.

4.2. Immunopathogenesis in post-streptococcal CNS disease

4.2.1. Theoretical possibilities of immunopathogenesis

There are a number of different possible mechanisms by which beta-haemolytic streptococcus can induce neuronal damage or dysfunction. The different mechanisms are summarised below:

Figure 4.1. Possible immune mechanisms in post-streptococcal CNS disease.



Clearly some of these mechanisms are considered less or un-likely. For example there is no evidence that intact streptococci gain access to the CNS in post-streptococcal CNS disorders. Furthermore, the fact that a large proportion of patients make a full recovery without any apparent brain damage makes T-cell injury and associated cytotoxicity less likely (although not impossible). Although a large number of

mechanisms are theoretically possible, most attention has focussed on B-cell / antibody theories. It should be mentioned that a number of different immune mechanisms might be occurring concurrently.

4.2.2. Pathology

As post-streptococcal CNS syndromes are rarely fatal, pathological examination has been limited to a few case reports and case series. By virtue of their fatal nature, it is possible that these post-mortem descriptions may represent the more severe end of the spectrum. It is also possible that some of the early reports may have mistakenly included cases of unrecognised genetic or neurodegenerative disorders such as Huntington's disease or metabolic disease.

The histological findings can be separated into three main groups:

1. Inflammatory (encephalitic) features
2. Degenerative features
3. Thromboembolic features (rarely)

Table 4.c reviews the reports and histological features of SC (there are no pathological reports of PANDAS). In view of the conflicting features (inflammatory or degenerative), some authors have suggested that the inflammatory features are typical of the acute disease, whereas degenerative features are more typical of subacute or chronic disease. Alternatively, some authors have suggested that Sydenham's chorea is a syndrome only, with a number of different pathological processes causing the SC syndrome.

In the inflammatory cases, the most consistent finding is of encephalitis, with perivascular small cell infiltration predominantly of the basal ganglia and to a lesser extent the cortex (Greenfield JG and Wolfsohn JM, 1922; Marie P and Tretiakoff C, 1920). The caudate and putamen were usually the most severely involved basal ganglia regions (Greenfield JG and Wolfsohn JM, 1922; Marie P and Tretiakoff C,

1920). Perivascular infiltration by lymphocytes (particularly small round cells-probably plasma cells) was characteristic in these descriptions.

Table 4.c. Summary of pathological reports of SC.

Reference	Predominant feature	Detail
Colony HS et al., 1956	Degenerative	Cortex and thalamus
Lewy FH, 1923	Degenerative and inflammatory	-
Marie P and Tretiakoff C, 1920	Inflammatory	Perivascular inflmmation of basal ganglia
Greenfield JG and Wolfsohn JM, 1922	Inflammatory	Perivascular inflammation of basal ganglia and cortex.
Ziegler LH, 1927	Degenerative	-
Gordon RG and Norman RM, 1935	Degenerative	-
Lhermitte Jand Pagniez P, 1930	Inflammatory or Degenerative	-
Van Bogaert L and Bertrand I, 1932	Inflammatory or Degenerative	-
Von Santha K, 1932	Vascular	'Rheumatic encephalitis'
Glaser GH, 1952	Vascular	Reminiscent of CNS lupus
Lehoczkya TV, 1941	Inflammatory, degenerative or vascular	-
Delcourt and Sand 1908	Inflammatory (16 cases)	Perivascular inflammation of basal ganglia and cortex
Guizzetti and Camisa, 1911	Inflammatory and vascular (n=2)	Disseminated encephalitis with secondary ischaemia of cortex
Harvier and Levaditi, 1920	Inflammatory	Similar to encephalitis lethargica. Perivascular inflammation of mesencephalon.

4.2.3. Auto-antibodies in post-streptococcal CNS disease

4.2.3.1. Sydenham's chorea.

Support for the antibody hypothesis was first demonstrated by Husby who described anti-neuronal antibodies using an immunofluorescent technique in 46% of Sydenham's chorea patients (n=30), compared to 14% of rheumatic fever (without chorea n=50), and only 1.8-4% of control subjects (n=203) (Husby G et al., 1976). The antibodies demonstrated a cytoplasmic pattern of binding to caudate and subthalamus neurones, with occasional weaker staining in the cortex and medulla. Furthermore, Husby demonstrated a potential correlation between antibody reactivity and the clinical status, with antibody disappearance on chorea remission. In addition, the antibodies were removed by pre-incubating with a preparation of isolated caudate neurones, but not cerebral cortex or mouse liver, supporting antibody specificity to caudate neurones (Husby G et al., 1976). Two further studies have expanded upon these findings; both demonstrated antibodies reactive against basal ganglia neurones universally in acute Sydenham's chorea (100%), although less commonly in the chronic or persistent stage (63%) (Church AJ et al., 2002; Kotby AA et al., 1998; Morshed SA et al., 2001). A further study using immunofluorescence showed increased IgG to neuronal tissue in SC compared to normal controls (Morshed SA et al., 2001). Although antibody assays using immunofluroescence were important in establishing a putative antibody hypothesis, Western immunoblotting studies by Church have suggested a conserved group of autoantigens are involved in auto-antibody binding (Church AJ et al., 2002). Andrew Church developed this method at the Department of Neuroinflammation, Institute of Neurology. The antigen was post mortem human caudate and putamen from the Queen Square brain bank. The antigen was homogenised and delipidated before being solubilised with sodium dodecyl sulphate. The assay was protein standardised and controls run on each blot (Church

AJ et al., 2002). The Western blotting suggested that a conserved group of brain proteins were involved in autoantibody binding. 100% of the acute SC group (n=20) had positive Western blotting binding compared to 69% of the persistent SC group (n=16), 13% of the RhF group (n=16) and 0% of the healthy group (n=11). In the acute SC group there was binding to a 40 kDa protein in 50% of patients, 45 kDa protein in 50% of patients and 60 kDa protein in 40% of patients. Only 15% had no reactivity to any of these antigens (Church AJ et al., 2002). The persistent SC group had similar autoantigens (Church AJ et al., 2002). Although these were the predominant proteins, there were other autoantigens of 30kDa protein in 20% of patients, 50 kDa protein in 25% of patients, 80 kDa in 30% of patients and 95kDa protein in 25% of patients. The two rheumatic fever patients with positive binding had autoantibodies to the 40 kDa protein. Church's conclusions were that the 40, 45 and 60 kDa brain antigens were discriminating autoantigens and possibily significant in the pathogenesis of post-streptococcal CNS disorders. Using the same methods, but in a separate SC cohort, Church found 93% (13/14) of acute SC patients had auto-antibodies to brain proteins, again binding predominantly to the 60 kDa protein in 50% of patients, 40 kDa protein in 36% of patients and 45 kDa protein in 21% of patients (Church AJ et al., 2003). The primary immunological aim of this thesis (as discussed later) is to identify these brain antigens using proteomic techniques. A further small study by a separate group also proposed that a 45 kDa was found in SC, although only during the acute stage (Frucht S et al., 1997).

Singer performed a similar examination for anti-neuronal antibodies in SC again using human basal ganglia (Singer HS et al., 2003). He separated supernatant, pellet and synaptosomal fractions and found elevation of the mean optical density (using ELISA) in all three fractions in the SC versus control group, although no statistical difference was achieved. There was no difference using adult and paediatric brain

tissue as the autoantigen. Western blotting was also performed although enhanced chemiluminescence was used (a notoriously sensitive method) which revealed a plethora of auto-antibody bands in patient and control samples, the majority of which were unlikely to have been relevant. Singer had to use discriminant analysis using ScanPack images to detect differences between the SC and control cohorts (Singer HS et al., 2003). Although there were clear differences between the SC and control cohort, a number of autoantigen molecular weights were proposed as being significant (amongst them a 44 kDa autoantigen) (Singer HS et al., 2003).

4.2.3.2. PANDAS

Church measured anti-neuronal antibodies in 16 patients with post-streptococcal tics conforming to PANDAS criteria (Swedo SE et al., 1998). He compared the findings with 190 control samples (children with neurological disease, recent uncomplicated streptococcal infection, autoimmune diseases). Positive Western blotting was present in 15/16 (94%) PANDAS patients, compared to only 8/190 (4%) controls (Church AJ et al., 2004). Again, the positive autoantibody binding was to the same as that observed in SC, to brain autoantigens of molecular weight 40, 45 and 60 kDa.

Pavone examined the sera of 22 Italian patients with Swedo's diagnostic criteria for PANDAS (Pavone P et al., 2004). They ranked antibody positivity using an immunofluorescent technique as described by Husby, and compared them with 22 patients with confirmed GABHS tonsillitis but no neuropsychiatric features. Pavone found 64% of the PANDAS patients had positive anti-neuronal antibodies compared to 9% of the GABHS group ($p<0.001$ Fisher exact test).

Singer performed ELISA and Western blot analysis for anti-neuronal antibodies in 15 PANDAS patients compared to 15 controls (Singer HS et al., 2004). Again Singer used the same methods as used in his SC cohort including ECL development and

ScanPack interpretation with discriminant analysis (Singer HS et al., 2004). ELISA showed no difference in the means between the groups. Western blotting showed complex staining patterns with antibodies directed at multiple molecular weight regions in patients and controls. Discriminant analysis revealed differences between the PANDAS and controls only using the caudate supernatant antigen preparation, the difference most noticeable in the tic rather than OCD PANDAS patients. Singer's conclusions, in contrast to Church's conclusions, were that anti-neuronal antibodies are not elevated in PANDAS, nor are there clear autoantigens in PANDAS (Singer HS et al., 2004).

4.2.3.3. Other post-streptococcal CNS syndromes.

As part of this thesis, I describe a broader clinical spectrum of post-streptococcal CNS disorders including post-streptococcal acute disseminated encephalomyelitis (with dystonia), post-streptococcal dystonia and post-streptococcal Parkinsonism (Dale RC et al., 2001, Dale RC et al., 2002a, Dale RC et al., 2002b; Dale RC et al., 2004). Using Church's methods these patients also had elevated anti-neuronal antibodies compared to controls. Again, the anti-neuronal antibodies bound to autoantigens of molecular weight 40, 45 and 60 kDa (Table 3h). In the Parkinsonism cohort, there was an additional band to a 98 kDa autoantigen.

4.2.3.4. Anti-neuronal antibodies in Tourette syndrome and obsessive-compsulive disorder

As anti-neuronal antibodies are the proposed mediators of SC and PANDAS, there have been cross-sectional studies examining the serum of patients with Tourette syndrome for anti-neuronal antibodies. The results have been mixed and conflicting. Although some studies have demonstrated more prevalent anti-neuronal antibodies in TS (Singer HS et al., 1998; Church AJ et al., 2003; Wendlandt JT et al., 2001), other

studies have failed to find an association (Singer HS et al., 1999; Loiselle CR et al., 2003; Morshed SA et al., 2001). It is of interest that the three positive studies have proposed a basal ganglia protein of 60 kDa to be the most discriminating antigen in TS compared to controls (Singer HS et al., 1998; Church AJ et al., 2003; Wendlandt JT et al., 2001). We have reported that this 60 kDa antigen appears to be common to both SC and Tourette syndrome (TS), supporting the hypothesis that post-streptococcal autoimmunity may also be important in TS (Church AJ et al., 2003). Hoekstra identified a 60 kDa antigen as heat shock protein 60 (Hoekstra PJ et al., 2003). This is a ubiquitous heat shock protein, although a homologous protein exists on the streptococcal organism. However, the protein purification used by Hoekstra was possibly inadequate, as the brain homogenate proteins were only separated using 1-dimensional PAGE electrophoresis, and the candidate protein cut from a coumassie gel. It is probable that this one stage purification resulted in inaccurate results. Hoekstra was able to demonstrate increased reactivity in the Tourette patients compared to controls (67% in Tourette patients compared to 40-42% reactivity in control groups) (Hoekstra PJ et al., 2003). It is acknowledged that longitudinal studies comparing clinical, microbiological and immunological markers are essential to define whether a true association exists in TS and OCD (Loiselle CR et al., 2003).

4.2.4.Cross-reactivity of antibodies with streptococcal and brain proteins

Although streptococcal organisms are the proposed mediators of SC and PANDAS, relatively little attention has focussed on why beta-haemolytic streptococcus is capable of producing immune-mediated brain disease. The favoured hypothesis is that antibodies cross-react between streptococcal and brain epitopes (molecular mimicry). Immunization of rats with M6 streptococcal proteins has been shown to induce cross-reactive anti-brain antibodies (Bronze MS et al., 1992; Bronze MS and Dale JB,

1993). Furthermore, synthetic epitopes of M6 protein sequences were capable of inhibiting anti-brain antibodies from a patient with Sydenham's chorea. Husby's original experiments suggested that the antibodies against caudate neurones cross-reacted with epitopes of Group A streptococcal membranes (Husby G et al., 1976). It would also appear that the antibody cross-reactivity is specific to certain strains of streptococcus (Dale RC et al., 2001; Husby G et al., 1976; Bronze MS and Dale JB, 1993).

A recent and exciting development was the first attempt to directly link antibody cross-reactivity between specific streptococcal and neuronal antigens. Kirvan et al. took peripheral blood lymphocytes from patients with SC and created hybridomas and therefore monoclonal antibodies (Kirvan CA et al., 2003). One monoclonal antibody showed strong reactivity with N-acetyl-beta-D-glucosamine (GlcNAc), a Group A streptococcus carbohydrate moiety on the streptococcal cell wall. Using competitive inhibition assays (ELISA and immunofluroescence), they demonstrated that these antibodies also bound to lysoganglioside GM1, a dominant brain ganglioside present on the neuronal membrane. This group also showed that the antibodies bound to the neuronal membrane resulting in increased intracellular signalling via CaM kinase II activation (76% above the basal level). They also showed that acute SC serum and CSF could also activate CaM kinase II compared to controls. In summary, this group demonstrated a direct link between a streptococcal carbohydrate moiety and neuronal proteins resulting in altered neuronal cell signalling (Kirvan CA et al., 2003). The relevance to a broad spectrum of patients with post-streptococcal CNS syndromes remains as yet untested.

4.2.5. Animal model- passive transfer of IgG

The presence of serum anti-neuronal antibodies does not infer pathogenicity. Indeed auto-reactive antibodies against host tissue are common in healthy individuals. For an auto-antibody to be pathogenic, it must be possible to demonstrate effector function. One important method to establish effector function is the induction of disease in an animal after passive transfer of antibody. There has been three such animal models published in the literature to date that are summarised in table 4.d.

Table 4.d. Passive transfer of immunoglobulin to animals.

Paper	Animal	Infusion	Observation	Outcome
Hallett JJ et al., 2000	Rat	IgG or serum of TS (n=5) or control (n=5).	Stereotypical movement or behaviour	More Utterances in TS rats compared to controls
Taylor JR et al., 2002	Rat	Serum of TS with raised anti-neuronal antibodies (N=12), TS (n=12), control (n=12).	Oral stereotypy	Sig. higher oral stereotypies in rats given TS serum with raised anti-neuronal antibodies.
Loiselle CR et al., 2004	Rat	Serum of TS (n=10), PANDAS (n=10), control (n=14).	Stereotypic behaviour	No difference

TS: Tourette syndrome

PANDAS: Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections.

All three studies infused IgG or serum directly into the striatum using microcannulae. Two studies were positive with induction of stereotypical movements or oral stereotypies in the index animals compared to controls. However, these findings were not reproduced in the third (Loiselle CR et al., 2004) study. The Hallett study

performed immunohistochemistry after the infusion that showed IgG binding to neurone membrane in the index cases with secondary internalisation of IgG into the cell (Hallett JJ et al., 2000). It should be noted that the papers used different patient serum: some used patients with Tourette syndrome, some with PANDAS. Likewise the definition of the presence of serum anti-neuronal antibodies differed in the three studies: Hallett used an ELISA against a neuroblastoma cell line homogenate (Hallett JJ et al., 2000), Taylor used an immunofluorescence technique against rat striatum (Taylor JR et al., 2002), and Loiselle used ELISA with human putamen synaptosomes (Loiselle CR et al., 2004). In summary there is some evidence that patients with Tourette syndrome have IgG that can induce brain dysfunction compatible with Tourettism in animals. Clearly it would be preferable to repeat these experiments using anti-neuronal antibodies against clearly defined neuronal antigens (rather than polyclonal serum or IgG) to further examine the pathogenicity of IgG using animal models.

A further recent animal model used a different approach. Rather than using patient IgG, they immunised mice with a homogenate of Group A streptococcus (GAS). Specifically, an M6 streptococcus was homogenised and immunised serially in mice with Freund's adjuvant (Hoffman KL et al., 2004). The study was controlled with mice immunised with Freund's adjuvant only. The GAS immunised mice had serum IgG that bound to neuronal tissue, particularly in the deep cerebellar nuclei (DCN), although to a lesser extent in the basal ganglia (in addition, post-mortem immunohistochemistry of the mice brains showed IgG deposits that were present in the DCN of the GAS immunised mice only). The mice with DCN IgG were more likely to have behavioural abnormalities, particularly rearing or repetitive behaviours. This group also showed that the GAS antibodies cross-reacted with cerebellar proteins subsequently identified as complement (C4) and alpha-2 macroglobulin. This study is

the first animal model showing that GABHS can cause immune-mediated brain disturbance. Criticism of this study would include:

- The failure to control the study with a different micro-organism.
- Mice are not natural hosts for GAS, therefore their immune response may differ from humans.
- The cerebellum was primarily involved rather than the basal ganglia.
- Homogenisation of the streptococcus may destroy or create immunogenic epitopes.
- Many of the behavioural disturbances could be induced by cytokines resulting in sickness behaviour.
- Complement C4 and alpha-2 macroglobulin are ubiquitous proteins, not brain specific.

4.2.6. Plasmapheresis in post-streptococcal CNS disease

Improvement in clinical symptoms after plasmapheresis is another requirement to demonstrate antibody pathogenicity. The only controlled trial of plasmapheresis has been presented in the treatment section of this introduction. This small study demonstrated a benefit of plasmapheresis on clinical symptoms supporting an autoantibody pathogenesis (Perlmutter SJ et al., 1999).

4.2.7. Other immune studies

There have been relatively few other immune related investigations in post-streptococcal CNS syndromes. Related to anti-neuronal antibodies, Church measured the presence of oligoclonal IgG in the CSF. He found 46% (6/13) of acute SC patients had abnormal patterns of oligoclonal IgG in the CSF (2 intrathecal synthesis, 4 a

mirrored pattern) (Church AJ et al., 2003). Church also demonstrated that the IgG subclass involved in anti-neuronal antibody binding were of IgG1 and IgG3, not IgG2 or IgG4.

Cytokines are important markers of immune activation. Th1 cytokines (interferon-gamma etc.) are pro-inflammatory, whereas Th2 cytokines (interleukin-4, interleukin-10, etc) are anti-inflammatory cytokines. Interferon-gamma was undetectable in the CSF of acute or persistent SC, whereas IL-4 and IL-10 were modestly elevated in the CSF of some SC patients (Church AJ et al., 2003). These findings support a Th2 cytokine profile, and the authors concluded that this would support an autoimmune, autoantibody mediated hypothesis. The absence of IFN-gamma makes a cell-mediated pathophysiology less likely. Clearly there are a large number of other immune related investigations still required in post-streptococcal CNS disease, including the role of T-cell help and the superantigen mediated hypothesis.

4.2.8. Genetic and disease predisposition.

The mechanism of the genetic predisposition in post-streptococcal CNS disease is not certain; classical HLA class I and II profiles in SC do not appear to predict a genetic vulnerability (Donadi EA et al., 2000). Instead, interest has focussed on a B-lymphocyte marker (D8/17) that is highly expressed in patients with rheumatic fever/SC compared to healthy controls and autoimmune controls (including post-streptococcal glomerulonephritis) (Gibofsky A et al., 1991; Khanna AK et al., 1989). This same marker is significantly more prevalent in PANDAS (post-streptococcal tics and OCD) patients (Swedo SE et al., 1997), supporting immunological similarity between SC, rheumatic fever and PANDAS (table 4.e.). As can be seen, D8/17 is not only more prevalent in RhF and SC, but also in idiopathic tics and OCD compared to

controls (Table 4.e.). Despite this intriguing finding, the function of this lymphocyte marker remains unknown.

Table 4.e. D8/17 expression in SC, PANDAS and other neuropsychiatric syndromes. Percentage of B-cell lymphocytes (DR) expressing D8/17 on surface. All studies yielded statistically significantly differences.

Definition of positive (% of cells staining for D8/17	Patient group (number)	Percentage of patients positive	Control group (number)	Percentage of controls positive	Reference
>12%	SC (n=9)	89%	Healthy (n=24)	17%	Swedo SE et al., 1997
>12%	PANDAS (n=27)	85%	Healthy (n=24)	17%	Swedo SE et al., 1997
>11.8%	RhF (n=84)	99%	Healthy (n=76)	14%	Khanna AK et al., 1989
>11.8%	TS/OCD (n=31)	100%	Healthy (n=21)	5%	Murphy TK et al., 1997
95% percentile of controls	Tic disorders (n=33)	60.6%	Healthy (n=20)	5%	Hoekstra PJ et al., 2001

TS: Tourette syndrome
OCD: obsessive-compulsive disorder
SC: Sydenham's chorea
PANDAS: pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections
RhF: rheumatic fever

4.3. Immunological aims of this thesis

The immunological aims of this thesis are as follows:

1. To learn protein purification and immunoblotting techniques.
2. To use these methods to purify candidate autoantigens from brain homogenates.

3. To use these methods to identify the 40 kDa, 45 kDa and 60 kDa (and 98 kDa) autoantigens involved in autoantibody binding in post-streptococcal CNS disease.

Chapter 5. Protein purification and immunology methods.

Protein purification

5.1. Tissue preparation for Western immunoblotting

5.1.1. Human basal ganglia

Human striatum (caudate and putamen) were excised from the donor within 48 hours post mortem. The tissue was then frozen at –80°C until use. All human brain tissue was kindly provided by the Queen Square brain bank.

5.1.2. Rat brain and rat liver, kidney, heart

Adult Wistar male rats were used for all Western immmunoblotting using rat tissue. Rats were reared at B&K, Hull, UK. The brains and other organs were removed within minutes of death and snap frozen in liquid nitrogen. The brains and other organs were transferred to Institute of Neurology frozen within 24 hours of death, and stored at -80°C until use.

5.1.3. Tissue homogenisation method

1. The Teflon homogeniser and plunger were cleaned adequately and dried before use.
2. The whole procedure was done on ice. The brain was added to the homogeniser after cutting into smaller pieces. T-PER (tissue protein extraction

reagent) was added at 1ml per gram of tissue. Protease inhibitor cocktail for mammalian tissue was added at 100µl per 2g of tissue.

3. The tissue was homogenised with the plunger for 10 minutes and until the tissue was fully homogenised.
4. The homogenate was pipetted into tubes and centrifuged at 10,000g for 10 minutes.
5. The supernatant was pipetted into fresh tubes and re-spun until the supernatant was clear. The cell debris was discarded.
6. The homogenate was frozen in fractions until use.

5.2. Polyacrylamide gel electrophoresis

5.2.1. Introduction

Polyacrylamide gels can be purchased pre-cast in plastic housing. Polyacrylamide gels are formed by polymerisation of acrylamide monomers into long chains, followed by cross-linking these chains with compounds such as N, N-methylene-bisacrylamide (bis). This system creates a porous gel. The ability of a protein to pass through the pores depends upon the size of the proteins to be separated. I generally used the pre-cast polyacrylamide gel electrophoresis (PAGE) gels from Invitrogen for protein separation and Western blotting. The PAGE gels come in Bis-Tris gels (protein range 1.5-300 kDa) or the Tris-Acetate gels (30-400 kDa). I have generally used the Bis-Tris gels. The Bis-Tris gels come in 4-12%, 10% or 12%. These gels differ in their acrylamide concentration that generally influences the gel pore size and therefore migration. The higher concentration gels allow better separation of middle and lower molecular weight proteins. The buffers that are used influence the abilities of proteins

to migrate (discussed later). Most PAGE systems now use a detergent system for the sample such as sodium dodecyl sulphate (SDS). If the sample is run in native form, the proteins will be separated by charge and size. Insoluble proteins also aggregate and block the pores of the electrophoresis system resulting in poor results. For this reason, the SDS system is now widely employed. SDS (or lithium dodecyl sulphate-LDS) strongly binds to proteins and saturates the protein with approximately 1 SDS molecule per 2 amino acids. The SDS denatures the protein and unfolds the protein into a polypeptide. In addition, the protein is saturated with a negative charge, thereby abolishing the resident charge of the protein, and instead replacing it with a negative charge. The proteins, once loaded onto the top of the gel (in a vertical system) migrate into the gel by creating a vertical charge through the gel (bottom of the gel has a positive charge, top of the gel has a negative charge). Therefore the protein is then separated only by the protein size, and not charge (the charge/size ratio is virtually identical for all proteins when in SDS/LDS). This therefore gives a relatively accurate guide to the protein's molecular weight. In addition, a small amount dithiothreitol (DDT) 0.05 Molar is added as a reducing agent, that disrupts any disulphide binds. This results in further denaturing of the protein, and sometimes separates protein subunits.

5.2.2. Polyacrylamide gel electrophoresis method in detail

1. The sample to be run was prepared as follows: 25% LDS sample buffer, 10% of 0.5Molar DDT. The sample, appropriately diluted in double distilled water is added and mixed thoroughly. The sample is then placed in the water bath at 65°C for 15 minutes.

2. The following running buffers can be used: NuPAGE 2-(N-morpholino) ethane sulphonic acid (MES) SDS running buffer. NuPAGE 3-(N-morpholino) propane sulphonic acid (MOPS) SDS running buffer. The buffer constituents are described in appendix 5. MES buffer was routinely used. MOPS buffer allows better separation of medium to lower molecular weight proteins (30-60 kDa) and was used when required. The buffer solutions are concentrates and were therefore diluted with distilled water before use.
3. The pre-cast PAGE gels were stored in the cold room. The gels were removed from their plastic wrapping, and the preservative was poured away. The plastic well guard and the tape from the bottom of the cassette were then removed. The well was washed with running buffer three times and loaded into the Mini-cell. The well should face toward the inner core. If two gels were to be run, the well of the second gel also faces into the core of the Mini-cell (so that the wells are open to the inner core). The gel tension wedges were inserted so that there is no leak of buffer from the inner chamber. The running buffer was poured into the inner core, and then the surrounding chambers. The buffer must be added so that the gel wells were completely covered by buffer. Bubbles, which congregate around the bottom of the cassettes, were removed by tilting the mini-cell.
4. Next the samples were prepared and loaded into the well. A molecular weight marker was loaded (4-5 µl) into lane 1 (See Blue Plus2 pre-stained standard, Invitrogen). The sample (now in SDS, DDT and heated) was loaded into the wells. In a 10 well gel, 25 µl was loaded per well. In a 2D well gel, 150 µl was pipetted into the well.

5. The loaded material was allowed to settle, and then the PowerEase electrophoresis apparatus was started. The following protocols were used, and found to produce good electrophoresis:

   Bis-Tris gel with MES running buffer

   - Voltage: 200V
   - Anticipated current start 110-125 mA/gel, end 70-80 mA/gel.
   - Run time 39 minutes

   Bis-Tris gel with MOPS running buffer

   - Voltage: 200V
   - Anticipated current start 100-115 mA/gel, end 60-70 mA/gel.
   - Run time 50 minutes

6. Once the electrophoresis was complete, the mini-cell was disconnected from the electrodes, and the running buffer was poured away. The gels were removed from the Mini-cell. The gel was then removed from the cassette. The cassette was laid onto paper with the well facing upwards. Using a knife to open the cassette, the top wall of the cassette was removed leaving the gel attached to the lower wall of the cassette. Then the cassette was turned over and the gel was manipulated off the cassette wall using the knife. The gel was then placed in a plastic tray for staining, or transferred to nitrocellulose for Western blotting.

5.3. Staining gels for NuPAGE Novex Bis-Tris 4-12% gels

5.3.1. Colloidal blue staining kit (Invitrogen)

1. After electrophoresis the gel was shaken in fixing solution (50% methanol, 10% acetic acid, 40% double distilled water) for 10 minutes.
2. The fixing solution was replaced with stainer A solution (20ml methanol, 55 ml double distilled water, 20 ml stainer A) and the gel shaken for 10 minutes.
3. Stainer B (5ml) was added to the solution and shaken for a minimum of 3 hours, maximum of 12 hours.
4. The staining solution was replaced with double distilled water and washed for 7 hours.
5. For short term storage, water was used. For longer term storage 20% ammonium sulphate solution was used (both at 4°C).

5.3.2. Silver staining (Amersham Biosciences)

1. After electrophoresis the gel was shaken in fixing solution (100ml ethanol, 25ml glacial acetic acid, 125 ml double distilled water) for 30 minutes.
2. The fixing solution was replaced with sensitising solution (75ml ethanol, 10ml sodium thiosulphate, 17g sodium acetate and 165ml double distilled water). Glutardialdehyde was not used, as this forms covalent bonds that interfere with mass spectrometry. The gel was shaken in solution for 30 minutes.
3. The gel was washed in distilled water for 15 minutes with 3 changes of water.

4. The gel was shaken in silver reaction (25ml silver nitrate 2.5% w/v, 225ml distilled water, 0.1ml formaldehyde 37% w/v) for 20 minutes.
5. Then the gel was washed in distilled water for 2 minutes with 2 changes.
6. To develop the gel developing solution (6.25g sodium carbonate, 250ml distilled water and 0.05ml formaldehyde) was used for 2-10 minutes.
7. To stop the reaction, EDTA (3.65g) in 250ml water was used (for several minutes).
8. The gel was then washed for 15 minutes with 3 changes of water.

5.4. Ammonium sulphate precipitation

5.4.1. Introduction

Ammonium sulphate precipitation is also known as 'salting out'. The technique separates proteins secondary to their hydrophobicity. Different proteins have varying solubilities in salt solution. The ability of a protein to remain soluble in salt solution depends on the amount of hydrophobic patches on its surface (side chains of Phe, Tyr, Trp, Leu, Ile, Met, Val). Water molecules become ordered around the hydrophobic side chains. As the salt concentration becomes higher, the freely available water molecules become scarce. The water molecules ordered around the hydrophobic side chains therefore are pulled off, and the water molecules are integrated into the salt solution. This exposes the hydrophobic surfaces, which want to interact with one another and therefore aggregate. It is important that the salt does not bind and interact directly with the proteins and destablise the protein (defined chaotropic). In this regard, ammonium sulphate is considered the most appropriate salt, for technical and economic reasons. It is important to keep the pH and temperature of the solution

constant so that the procedure is reproducible. In practical terms, ammonium sulphate precipitation is quite simple. The sample is added to a known amount of double distilled water and a small amount of Tris-HCl (to maintain pH). A known amount of ammonium sulphate is added to the solution, mixed thoroughly, and then centrifuged. The precipitate is then stored or re-solublised for further use. Further ammonium sulphate is then added to the solution and the procedure is repeated for the next fraction. The amount of ammonium sulphate to be added for each step was calculated by Scopes (Scopes RK, 1994).

5.4.2. Ammonium sulphate precipitation method in detail

1. All of this method can be scaled up for larger volumes of sample.
2. The supernatant rat brain fraction was prepared as previously described (method 5.1.).
3. 9635 µl of double distilled water was poured into a clean glass flask.
4. 315 µl of the sample was added plus 50 µl of Tris-HCl, making a total volume of 10,000 µl.
5. The solution was mixed thoroughly on the magnetic stirrer without allowing the solution to froth.
6. The solution was brought to 20% ammonium sulphate concentration by adding ammonium sulphate (in grams) using Stokes ammonium sulphate precipitation table (in this case 1.13g). The solution was mixed thoroughly for 1 hour.
7. The solution was pipetted into centrifugation tubes and spun at 10,000g at 15°C for 20 minutes.

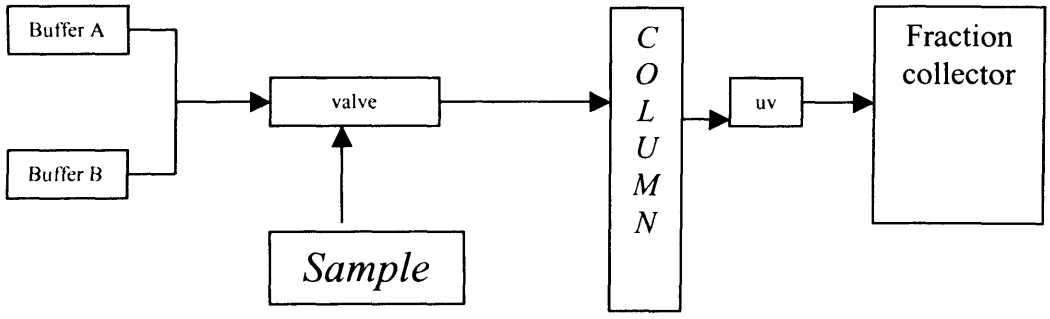
8. The resulting supernatant was then poured into the cleaned glass flask ready for the next addition of ammonium sulphate. The precipitate at the bottom of the centrifuge tube was stored or re-suspended in buffer ready for the next step (proteins are very stable in the precipitate).
9. Further ammonium sulphate was then added to the supernatant in the glass flask according the next desired ammonium sulphate fraction.
10. The resulting precipitates (0-20%, 20-40% etc) can be desalted into the buffer required for the next purification step, or stored in the precipitated form.

5.5. Fluid phase liquid chromatography: general principles

5.5.1. Introduction

Fluid phase liquid chromatography (FPLC) allows separation and purification of proteins in the liquid phase. A FPLC system requires a pump, detection meter (UV meter), fraction collector and computer for analysis.

Figure 5.1. FPLC diagrammatic representation



I have used the AKTA FPLC machine and UNICORN software from Amersham Pharmacia Biotech.

5.5.2. General method

1. In the UNICORN programme, by clicking on manual, it was possible to perform different pump instructions. After each alteration, it was necessary to click on 'insert' and 'execute' in order to fulfil this instruction. Using this programme it was possible to alter the flow rate and gradient (% of buffer B) to be used. In order to load the flow loop with sample, it was necessary to use the flowpath-load pathway before loading the sample.
2. The valve received buffers A and B. When performing a gradient, the buffers were mixed before entering the valve. In addition, the valve received input from the flow loop. The output of the valve was to the column.
3. The columns used in FPLC were always Amersham Biosciences columns. The majority were HiTrap columns, either 1ml or 5ml columns. The columns were filled with preservatives and were washed and cleaned accordingly (20% ethanol or starting buffer). To avoid bubbles, the buffer was dripped into the top of the column before screwing the connector into the top of the column. The column stood vertically just above the UV meter.
4. The UV meter performed continuous monitoring for UV adsorption at 280nm for protein (Trp + Tyr band). It was possible to set the UV adsorption to a more sensitive lower wavelength, such as 220-205 nm.
5. The fraction collector allowed for the fluid to be collected in tubes using an automated tube holder. The fraction collector can be programmed according to the fraction size and speed. 144 tubes can be used in the collector.
6. The UNICORN programme was used to create a method template including the following:

Characteristic	Detail
Flow rate	0.1-5 ml/min
Start buffer conc (%B)	% of buffer B in starting solution
Equilibration	Length of equilibration in column volumes
Flow-through fraction size	Size (ml) of fractions in flow-through
Sample injection (empty loop)	Volume of sample to be injected (less than volume loaded)
Wash out unbound sample	Volume of buffer to wash away unbound proteins from column (in column volumes)
Gradient	The length of gradient (in column volumes) to linearly increase from 0% to x% of buffer B. It is also possible to create a step gradient, as opposed to a linear gradient.
Elution fraction size	Volume of fractions to be collected (0.1-5ml)
Re-Equilibrate	Number of column volumes (of %B) to re-equilibrate the column

7. Templates were created and used for the following: desalting, IgG purification, ion exchange, hydrophobic interaction. It was possible to modify these templates as required according to screening experiments.

5.6. Desalting and changing the buffer

5.6.1. Introduction

Removing excessive salt from a sample, or changing the buffer is an essential procedure when using chromatography or before 2-dimensional electrophoresis. I performed all desalting or buffer changing using the HiTrap 5ml Desalting columns (Amersham Biosciences) and the FPLC. In essence, these columns are sieves: they allow proteins over 5,000 Daltons in size to pass unhindered through the gel, whereas the gel withholds small solutes and molecules. The desalting columns are packed with the 'exclusion matrix' Sephadex G-25 Superfine. The matrix is cross-linked dextran

beads. The exclusion limit of 5,000 Daltons allows easy passage of proteins through the column. The columns can be used with solutions of a wide range of pH, alcohols and urea. In order to maintain good desalting, it is important to keep the sample volume below 1.5ml.

5.6.2. Desalting method in detail:

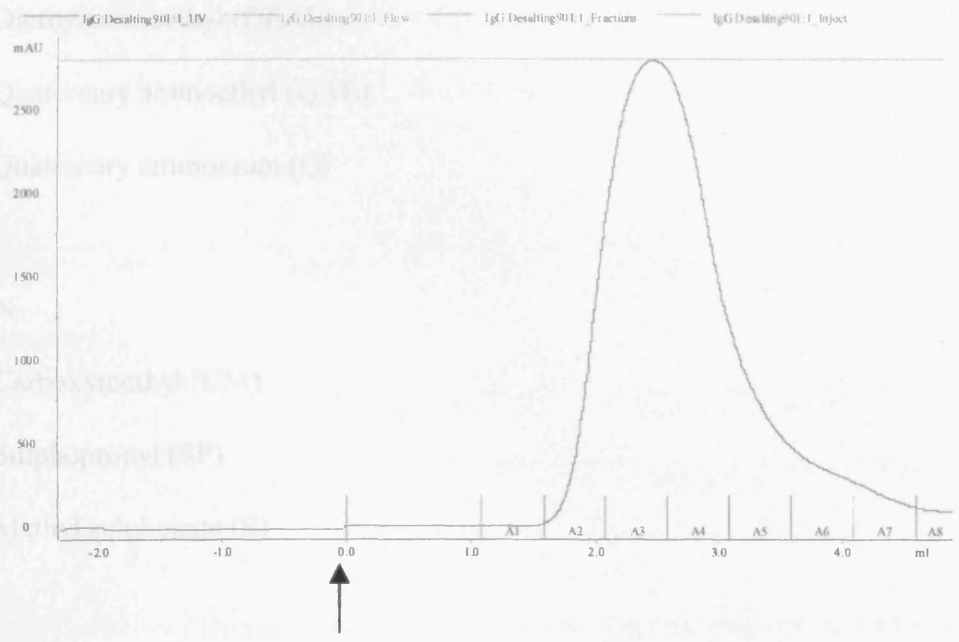
1. The FPLC was washed thoroughly with 20% ethanol and then equilibrated thoroughly with the starting buffer. The flow loop was also washed and equilibrated in the same manner.
2. A 5ml HiTrap Desalting column was removed from the cold room and attached to the FPLC. To avoid air bubbles, the column was connected with the 'drop-to-drop' technique, before the column was attached to the FPLC machine.
3. The starting buffer was run through the desalting column for at least 3 column volume until the UV meter reading was flat.
4. The sample was loaded into the flow loop. The sample volume should be no more than 1.5 ml (in a 2 ml flow loop). In addition, if the sample was from ammonium sulphate precipitation, the precipitate was first diluted with some starting buffer. Too high a salt concentration may impair desalting.
5. Then the FPLC was programmed as follows:

Flow rate	1ml/min
Equilibrate column	5 column volumes
Inject sample	<1.5ml
Fraction size	0.5ml

6. The sample was usually diluted by the procedure, approximately two-fold.

The typical sample chromatogram was as follows:

Figure 5.2. Desalting chromatogram



As can be seen, the sample was injected (arrow) and approximately 1.5ml later, the sample began to leave the column, now in the start buffer.

5.7. Ion exchange chromatography

5.7.1. Introduction

Ion exchange is one of the classic ways of separating proteins using chromatography. The technique uses ionic charge to separate proteins. The columns have a matrix that allows interaction of proteins with charged chains immobilised to the matrix. The matrix is composed of insoluble compounds such as dextran (Sephadex), cellulose (Sephacel) and agarose (Sepharose). Charged groups are covalently and irreversibly

bound to the matrix that give the column its exchange ability. The charged groups can be classified according to the charge (anionic or cationic):

ANION

- Diethylaminoethyl (DEAE)
- Quaternary aminoethyl (QAE)
- Quaternary ammonium (Q)

CATION

- Carboxymethyl (CM)
- Sulphopropyl (SP)
- Methyl sulphonate (S)

The charged groups are associated with 'counter ions' that are mobile and 'exchangeable'. Positively charged exchangers (anionic exchangers) have negatively charged counter-ions (anions) available for exchange, whereas negatively charged exchangers (cationic exchangers) have positively charged counter-ions (cations).

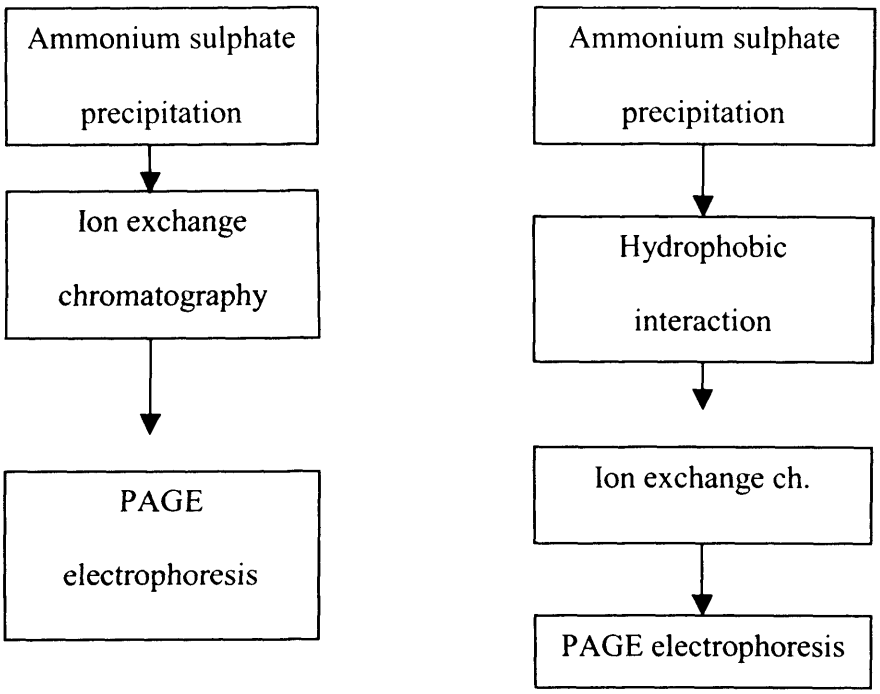
Before interaction with the column, the sample must be desalted into the start buffer. The choice of buffer is important. The protein of interest can be bound to the column and eluted, or alternatively the protein of interest could 'flow-through' the column without binding to the column. The buffer must contain acid or base to create the appropriate pH, and also contain an appropriate concentration of salt. Salts stabilise the proteins, and prevent precipitation (usually 10-20mmol). I have used citric acid (pH 2.6-3.6) for cation exchange, and Tris (pH 7.6-8.6) for anion exchange. It is

generally found that improved separations occur when the protein of interest is bound to the column and then eluted using a salt gradient (starting buffer plus 1 Molar sodium chloride). This achieves better separation than allowing the protein of interest to 'flow-through' the column without binding. The choice of column, and therefore buffer, depends on the charge (or isoelectric point) of the protein whilst in buffered solution. Using screening experiments, I have found that the following conditions have worked best:

Approximate isolectric point of protein (pI)	Column	Buffer
4-5	Cation (e.g. SP)	Citric acid (pH 3.0)
6-7	Anion (e.g. Q)	Tris (pH 8.0)

Using these conditions, the protein of interest elutes early on the gradient, and results in less contamination, although screening experiments are required. In order to ensure that the protein of interest binds to the column, it is important that the pH of the buffer is at least 1 pH unit below the pI of the substance to be bound (cation exchanger). Conversely using an anion exchanger, the buffer should be at least 1 pH unit above the pI of substance to be found e.g. for a protein pI 6.5, an anion exchanger with a buffer of pH 8.0 would allow binding to the column, and relatively early elution of the protein. I have generally found that proteins are more soluble and therefore achieve better separation using the anionic exchange system.

Ion exchange chromatography (IEX) can be used at different points in the purification procedure. I have used IEX at the following points during purification:



The decision to use these purification steps is dictated by a number of factors including:

- The yield of the protein to be purified
- The properties of the proteins. For example, a protein with an extreme pI such as 8.0 will be purified with less steps than a protein with a charge such as 6.0 (which is much more common).
- Contaminating protein properties.

5.7.2. Detailed method of ion exchange chromatography:

1. The appropriate buffer was chosen and freshly made. The elution buffer was made by pouring half of the starting buffer into a fresh glass container and adding 1Molar NaCl to the solution. The buffers was thoroughly mixed before use.

2. The sample to be purified was then desalted into starting buffer using the desalting columns as previously described.
3. The column and FPLC was washed thoroughly with 20% ethanol.
4. Then the column was washed with 5 column volumes of start buffer at 1ml/min (using the 1ml columns), or 3ml/min for the 5 ml columns. Then the column was washed with the elution buffer for 5 column volumes. This removed any residual contaminants from the column. Finally, the column was re-equilibrated with 5-10 volumes of the start buffer.
5. Next, the desalted sample was loaded into the flow-loop ready for injection.
6. Using the FPLC template to inject the sample, an elution gradient was produced as follows:

Step	**Detail**
Equilibration of column	5 column volumes
Volume of sample injected	Less than the volume of loaded sample
Wash after sample injection	At least 4 column volumes
Gradient	0-100% over 10-20 column volumes
Fraction size	0.5-1ml

The length of gradient, and the size of the fractions collected depended upon the degree of separation expected. For example, when the IEX was used early in purification, the length of elution was short and the fractions large, as the purification at this stage was crude. In comparison, if IEX was used as a late polishing step, a limited gradient (e.g. 0-40%) over 20 column volumes was employed with smaller

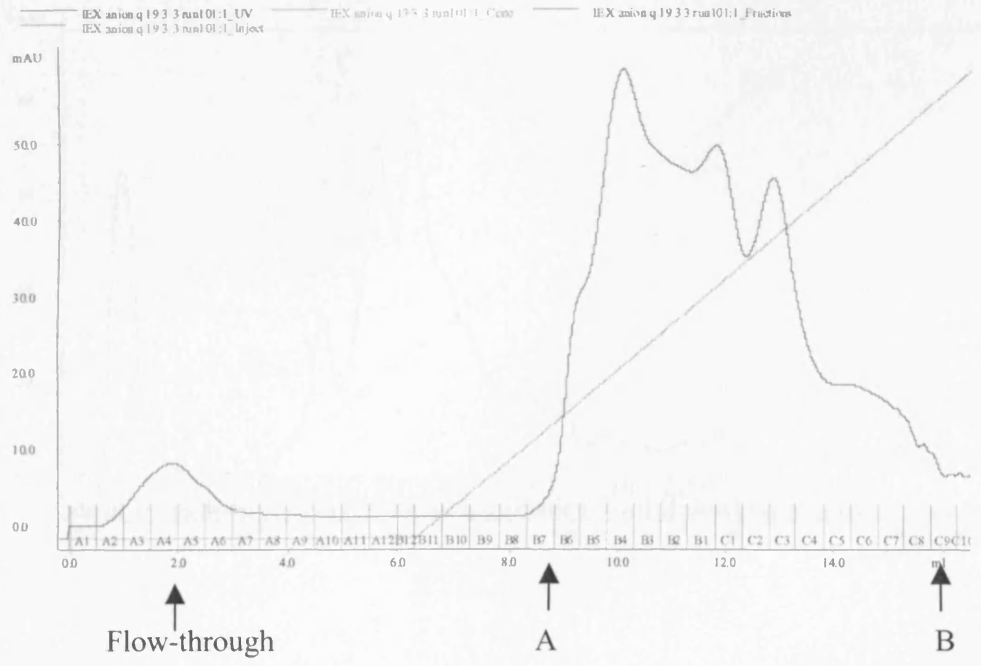
fraction sizes. Therefore the method varied slightly depending upon the aims of the IEX step.

The following chromatograms demonstrate the purification applications of IEX:

5.7.3. Screening experiment 1

Column	Mono Q 5ml
Sample	45-65% ammonium sulphate fraction of rat brain
Starting buffer	Tris (Ph 8.0)
Elution buffer	Tris (Ph 8.0) + 1Molar NaCl
Gradient	0-100% elution buffer over 10 column volumes

Figure 5.3. IEX screening experiment 1.

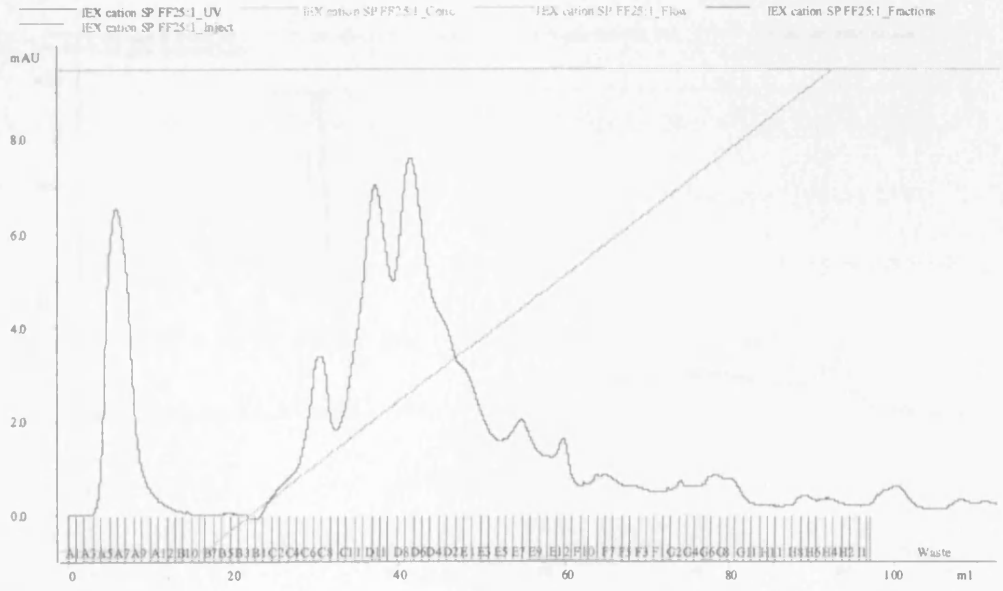


As can be seen, using this system, there was relatively good separation over the length of the gradient (between A and B). The IEX in this case was unlikely to produce adequate purification for autoantigen identification, unless further purification strategies are employed, such as hydrophobic interaction.

5.7.4. Screening experiment 2

Column	Mono Q 5ml
Sample	45-65% ammonium sulphate fraction of rat brain
Starting buffer	Tris (pH 8.0)
Elution buffer	Tris (pH 8.0) + 1Molar NaCl
Gradient	0-100% elution buffer over 80 column volumes

Figure 5.4. IEX Screening experiment 2.

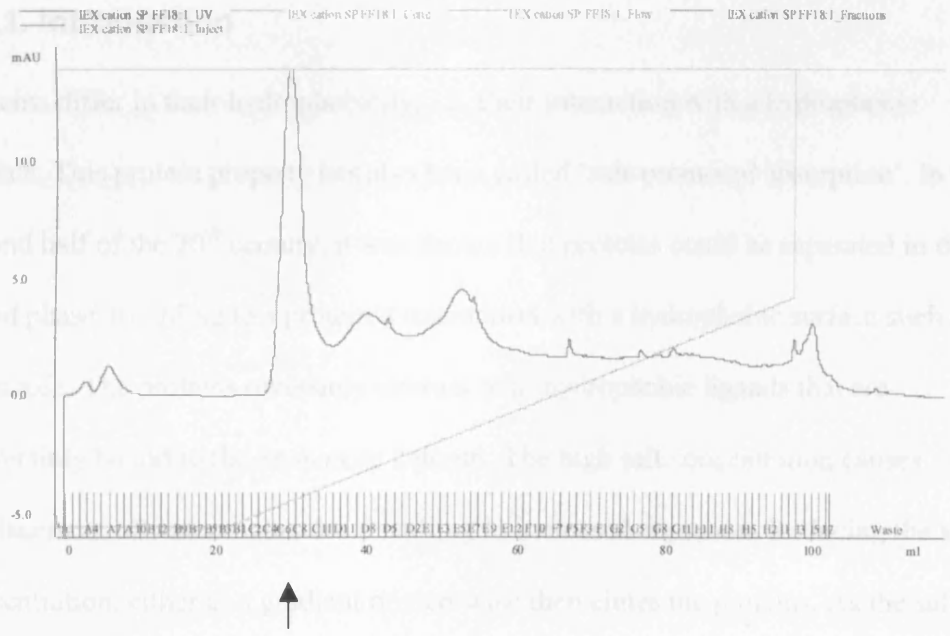


As can be seen, this time the gradient was lengthened significantly to 80 column volumes. By doing this, the column was able to equilibrate steadily, resulting in improved separation (sharpening of the peaks), although the proteins will be relatively diluted due to the prolonged elution.

5.7.5. Screening experiment 3

Column	Mono Q 5ml
Sample	1. 45-65% ammonium sulphate fraction of rat brain 2. Hydrophobic interaction chromatography
Starting buffer	Tris (Ph 8.0)
Elution buffer	Tris (Ph 8.0) + 1Moler NaCl
Gradient	0-50% elution buffer over 80 column volumes

Figure 5.5. IEX screening experiment 3.



As can be seen, this time I used IEX after ammonium sulphate precipitation and hydrophobic interaction chromatography. There were less contaminating proteins, and the protein of interest (arrowed) came off the column early in elution. IEX was used here as a polishing step, rather than an early cruder purification step. The gradient was long, and the fractions relatively small for the length of elution. The yield of the proteins (as suggested by the UV measurements) was low however.

5.7.6. Conclusion of Ion exchange chromatography method

In summary, IEX provides a very useful purification method. Indeed, I found IEX the most reliable and predictable purification method. The IEX method needed to be varied according to the charge of the protein to be purified.

5.8. Hydrophobic interaction chromatography

5.8.1. Introduction

Proteins differ in their hydrophobicity, i.e. their interaction with a hydrophobic surface. This protein property has also been called 'salt-promoted absorption'. In the second half of the 20^{th} century, it was shown that proteins could be separated in the liquid phase according to a protein's interaction with a hydrophobic surface such as sepharose. The proteins reversibly interact with hydrophobic ligands that are irreversibly bound to the Sepharose column. The high salt concentration causes displacement of water molecules, resulting in increased attraction. Reducing the salt concentration, either as a gradient or step-wise then elutes the proteins. As the salt concentration decreases, there is less 'shielding' of the protein by water molecules

resulting in increased hydrophobicity of the protein with its ligand, and elution of the proteins from the column.

The following variables effect hydrophobic interaction:

1. Immobilised ligands attached to the sepharose (aryl and alkyl). The preferred ligand (octyl, butyl, phenyl, alkyl) cannot be predicted, and screening experiments are necessary to determine which ligand will be best at separating proteins.
2. The salt used will alter the hydrophobic interaction. Classically sodium, potassium and ammonium salts have good 'salting-in' qualities, and are commonly used in HIC. The salt concentration needs to be established by screening experiments.
3. The pH will affect hydrophobic interaction, acidity generally increasing hydrophobic interaction. The pH must be maintained to preserve inter-assay comparisons.
4. Likewise, the temperature will affect hydrophobic interaction (either increase or decrease). For practical purposes, it is therefore necessary to maintain a consistent temperature to maintain reproducibity.

5.8.2. Method and screening experiments.

1. In the first instance the hydrophobic interaction chromatography (HIC media) test kit was purchased for initial screening (Amersham Biosciences). As phenyl sepharose has been used by previous investigators in the separation of

proteins from crude homogenates, I chose the HIC 1ml phenyl sepharose high performance column for initial screening.

2. The sample (30-70% ammonium sulphate fraction) was first desalted into the start buffer. The start buffer was sodium phosphate pH 7.0 buffer with added 1 Molar ammonium sulphate. The elution buffer was sodium phosphate pH 7.0 alone.
3. The column was attached to the FPLC and first equilibrated with 5 ml of elution buffer to remove any contaminants from the column. If this was inadequate, the column was eluted with distilled water, or 20% ethanol. After washing with the elution buffer, the column was equilibrated with 5-10ml of the start buffer (high salt buffer).
4. The sample (now desalted into the start buffer solution) was loaded onto the column.
5. The FPLC was then programmed using an 'HIC template'. The following programme was found to produce a reasonable chromatogram.

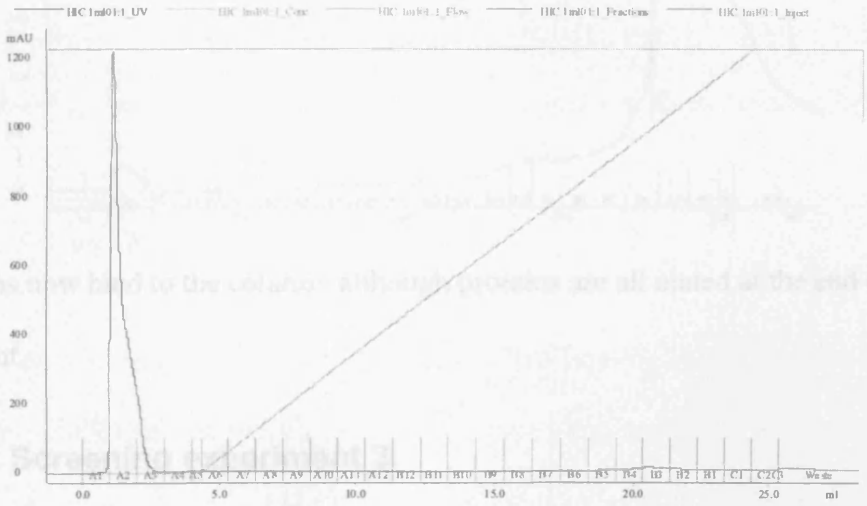
Variable	**Value**
Flow rate	0.25 ml/min
Wash out after sample injection	5 column volumes
Length of gradient	15 column volumes
Fraction size	1ml

6. After completion of the gradient, the column was regenerated with 5ml of distilled water and stored, or regenerated with the start buffer for a further sample.

5.8.3. Screening experiment 1

HIC was first performed with a 1 molar solution of ammonium sulphate in the sodium phosphate buffer. As can be seen in the chromatogram (Figure 5.6), this start buffer did not allow any proteins to bind to the column (i.e. form hydrophobic binding). All of the proteins flowed through the column without binding to the column, therefore failing to separate proteins.

Figure 5.6. HIC screening experiment 1 using: Start buffer: Sodium phosphate pH 7.0, 1 Molar ammonium sulphate. Elution buffer: Sodium phosphate Ph 7.0.



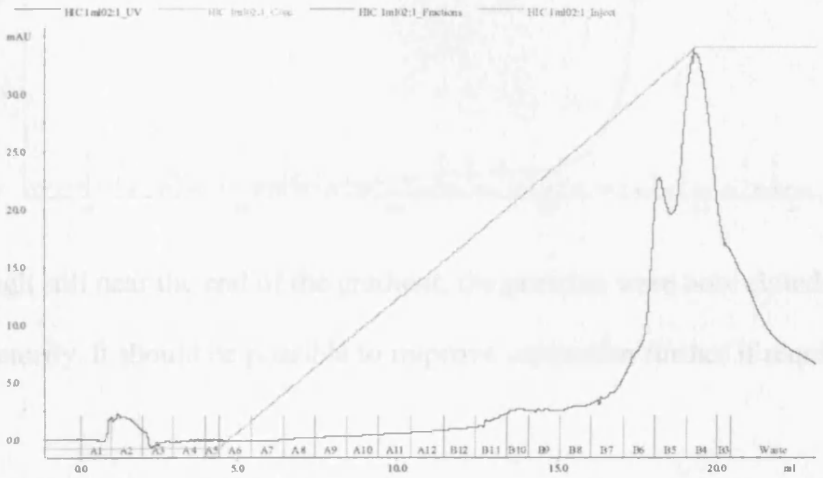
As can be seen from the chromatogram, all proteins flow through the column without binding to the column. I therefore needed to change the HIC conditions.

5.8.4. Screening experiment 2

In order to create a more hydrophobic environment, the salt conditions of the start buffer were increased to 3 molar ammonium sulphate in the sodium phosphate pH 7.0 buffer. This significantly improved binding to the column, although now the proteins

were so strongly bound, the proteins were eluted near the end of the gradient, including after completion of the gradient.

Figure 5.7. HIC screening experiment 2 finding using: Start buffer: Sodium phosphate Ph 7.0, 3 Molar ammonium sulphate. Elution buffer: Sodium phosphate Ph 7.0.

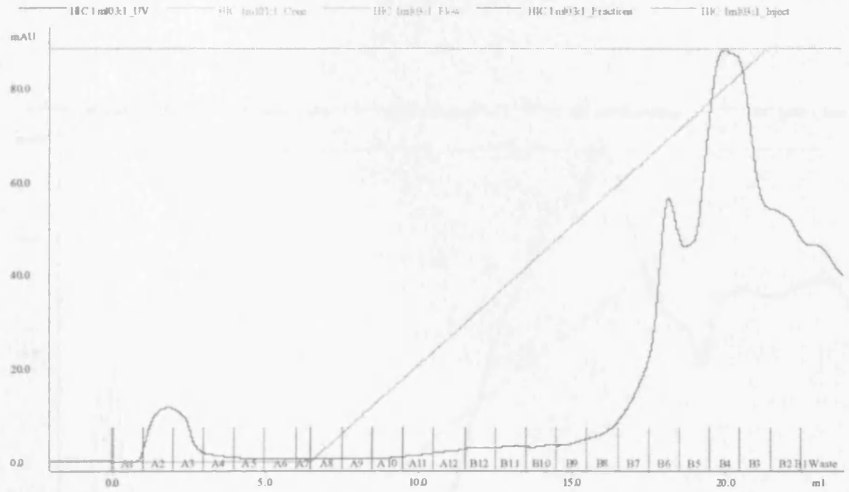


Proteins now bind to the column, although proteins are all eluted at the end of the gradient.

5.8.5. Screening experiment 3.

To allow proteins to be eluted towards the middle of the gradient, the salt solution was reduced to 1.5 Molar ammonium sulphate solution.

Figure 5.8. HIC screening experiment 3 using: Start buffer: Sodium phosphate Ph 7.0, 1.5 Molar ammonium sulphate. Elution buffer: Sodium phosphate Ph 7.0.



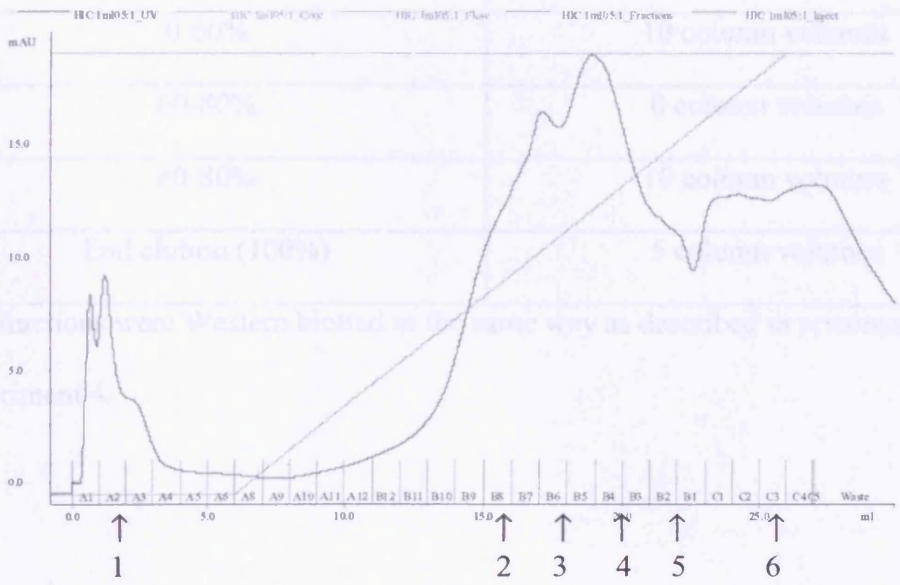
Although still near the end of the gradient, the proteins were now eluted more satisfactorily. It should be possible to improve separation further if required.

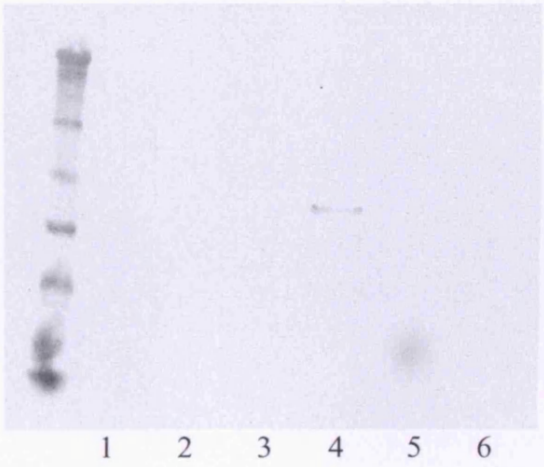
5.8.6. Screening experiment 4.

Despite alterations in the salt concentrations of the binding buffer, the chromatogram failed to significantly improve using the phenyl sepharose column. It is possible that an alternative column would allow improved protein separations. I therefore tried a different column from the HiTrap screening column series- the Butryl sepharose fast flow column. The FPLC and all of the variables were otherwise unchanged.

In order to determine the separation abilities of the butryl sepharose column, I Western blotted different fractions on a 4-12% Bis-Tris 10-lane gel. The protein fractions were normalised and transferred to nitrocellulose and Western blotted using the standard method. Serum from patient MB (anti-40 kDa antibodies) was used as the primary antibody. The blot was developed colormetrically (Figure 5.9).

Figure 5.9. HIC Screening experiment 4. Using this continuous gradient, the butryl sepharose column produced better interaction on the column, and improved elution over a larger range of the gradient. The 40kDa protein is present in fraction 4.





5.8.7. Screening experiment 5.

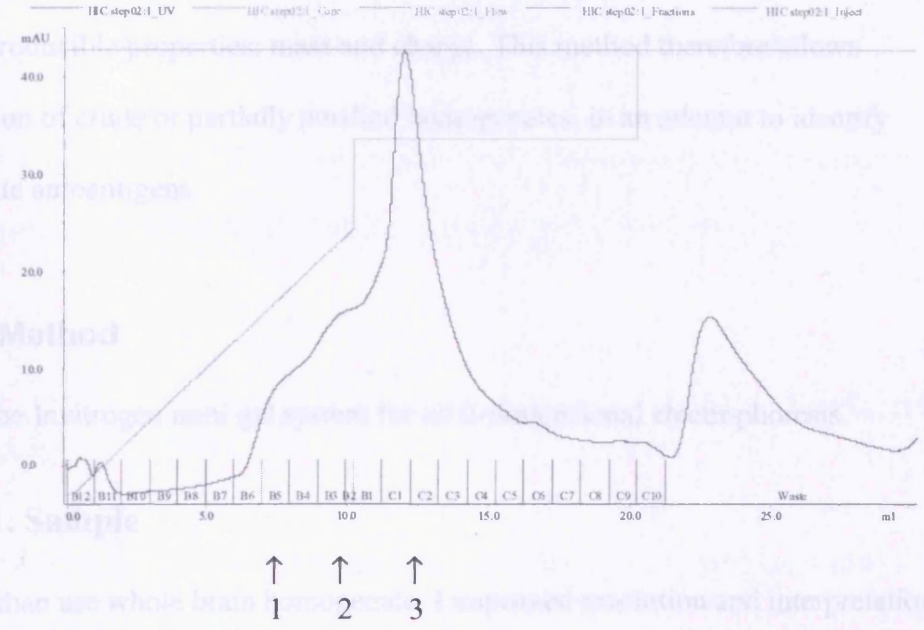
Using the Butryl sepharose column, I used a step gradient to see if the protein of interest could be eluted during a step. The 40 kDa protein eluted after 60% of the

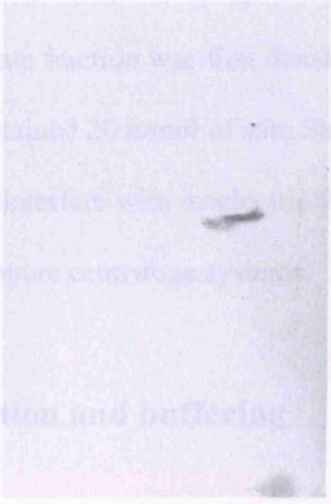
gradient, and before 80% of the gradient. I therefore used the same variables but introduced a step in the gradient as follows:

% buffer B	Length of elution
0-60%	10 column volumes
60-80%	0 column volumes
80-80%	10 column volumes
End elution (100%)	5 column volumes

The fractions were Western blotted in the same way as described in screening experiment 4.

Figure 5.10. HIC screening experiment 5. This STEP gradient allowed a large peak elution during the 60-80% step, which contained the 40 kDa protein. By introducing a step before the 60-80% step, it should be possible to minimise the contamination of the preceding proteins.





5.8.8. Conclusion

HIC may provide a useful purification step in conjunction with other purification strategies. The column and salt concentrations will need to be altered according to screening experiments (for each protein of interest).

5.9. 2-dimensional electrophoresis

5.9.1. Principle

2-dimensional electrophoresis employs a method of separating proteins according to two reproducible properties: mass and charge. This method therefore allows separation of crude or partially purified homogenates, in an attempt to identify candidate autoantigens.

5.9.2. Method

I used the Invitrogen mini gel system for all 2-dimensional electrophoresis.

5.9.2.1. Sample

Rather than use whole brain homogenate, I improved resolution and interpretation of 2-dimensional electrophoresis by first performing ammonium sulphate precipitation. The chosen ammonium sulphate fraction was first desalted into sodium phosphate buffer, Ph 7.0. The buffer contained 20 mmol of salt. Salt concentration significantly above or below this level can interfere with isoelectric focussing. The sample was then concentrated using micropore centrifuge systems.

5.9.2.2. Sample preparation and buffering

The electrophoresis demands that the proteins are soluble and expressing their native charge. However, a compromise needs to be met, as some proteins are poorly soluble in aqueous solutions and require small amounts of detergent to improve solubility. For this reason, a small amount of SDS was included in the sample preparation before

electrophoresis. Too much SDS could conversely impair separation. The sample was therefore made up as follows:

- 100 µl sample
- 25 µl 0.5% SDS
- 125 µl IEF sample buffer, Ph 3-10

This results in a 0.05% SDS concentration. The solution was mixed thoroughly, and left at room temperature for 20 minutes.

5.9.2.3. 1st stage electrophoresis (separation by charge)- isoelectric focussing

Using the Invitrogen/ NOVEX Power ease 500 systems, a pH 3-10 isoelectric focussing 10 well gel was employed.

The following buffers were required to create an appropriate charge current through the gel:

IEF Cathode buffer, pH 3-10

IEF Anode buffer, pH 3-10

The buffers were prepared and allowed to stand to remove bubbles.

1. The gel was removed from its packet and the preservative discarded. The wells were washed 3 times with the cathode buffer, and the gel was loaded onto the Mini-cell so that the well faces the buffer core. I completed the mini-cell and ensured the system was secure, and did not allow mixing of the inner and outer chambers.

2. I loaded the cathode buffer into the inner chamber, and ensured the buffer covers the well. I ensured the cathode buffer did not leak into the outer chamber.
3. I then loaded the anode buffer into the outer chamber.
4. Then I loaded 25 µl of sample (in sample IEF buffer and SDS) into each well (10 wells).
5. The gel was run for the following protocol:100 V constant (1 hour), 200 V constant-(1 hour), then 500 V constant (30 minutes). The approximate current is 5 mA/gel at the start, ending on 6 mA/gel. The protocol therefore lasts 2.5 hours.
6. I then removed the gel from the cast, and fixed the gel with IEF fixing solution for 30 minutes. This step fixed the proteins and removed the ampholytes. Then the gel was washed for 5 minutes in distilled water.
7. The gel was then stained for 5 minutes in Coomassie R-250 0.1%. This allowed visualisation of the lanes for the 2nd stage.
8. The gel was destained for 30-60 minutes to remove excess Coomassie.
9. After washing briefly, the gel was incubated with 20% methanol for 1 hour. This removed the acetic acid (destain solution) and shrunk the gel slightly to facilitate the 2nd stage transfer.
10. This completed the 1st stage. The gel was stored at +4^{o} C until use.

5.9.2.4. 2nd stage of 2-dimensional electrophoresis (polyacrylamide gel)

The next step separated proteins according to size.

1. I first cut out one lane of the IEF gel using a sharp straight blade.

2. The lane was then placed into an LDS solution (1ml LDS buffer, 1ml double distilled water, 0.5ml 100% methanol). The lane was incubated in this solution for 20 minutes with occasional gentle shaking.
3. The lane was then washed in MES running buffer and excess liquid was removed before transfer to 2-D NuPAGE gel.
4. The lane was trimmed slightly before transfer to the trough (pre-filled with MES buffer). For comparison between silver stained gel and Western blots, it was essential to be consistent in the extent of the trimming.
5. The lane was transferred with the aid of blotting paper that was cut to size. The blotting paper was just shorter than the width of the trough. The blotting paper was placed onto the gel segment, so that just over half the height of the gel segment is covered by blotting paper. This allowed the gel to be transferred to the trough. The lane must be inserted in a consistent way. The acidic end of the gel was always put in the lane next to the molecular weight marker. The lane was inserted and any bubbles removed before running.
6. 4 µl of molecular weight marker was loaded into the small chamber, and run using a standard PAGE electrophoresis protocol as previously described.
7. The gel was then stained using silver stain, or transferred to nitrocellulose for Western immunoblotting, as required.

5.10. Concentrating proteins

5.10.1. Introduction

For concentration of proteins in solution, it is possible to use centrifugation of solutions through membranes. Using the Amicon centrifugal filter devices

(Microcon), the membrane is sealed with a silicone rubber O-ring. The sample is placed on top of the membrane and, using centrifugation, the water and low molecular weight proteins pass through the membrane, retaining and therefore concentrating the larger molecular weight proteins. The low adsorption characteristic of the membrane means that >95% of proteins are recovered.

5.10.2. Concentration device method in detail

1. The Amicon centrifugal device (Microcon) to be chosen depended upon the molecular weight of the autoantigen of interest. The devices are named according to the membrane porosity. The following devices are applicable to the following autoantigen molecular weight
   - YM-3 (3,000 molecular weight limit)
   - YM-10 (10,000 molecular weight limit)
   - YM-30 (30,000 molecular weight limit)
   - YM-50 (50,000 molecular weight limit)
   - YM-100 (100,000 molecular weight limit)
2. The sample reservoir (with the membrane) was placed in the vial. The sample to be concentrated was then loaded into the sample reservoir (500µl at a time). The lid was closed securely and the assembly then placed in a fixed rotor centrifuge, with an appropriate counter-balance.
3. The assembly was then centrifuged at 10,000g for between 20-30 minutes. This allowed concentration to below 100µl.
4. Next the sample reservoir was removed from the vial, and the sample reservoir inverted into a new vial. Then the vial was centrifuged at 1,000g for 3 minutes,

propelling the concentrate into the new vial. Then the vial was removed from the centrifuge, the reservoir discarded, and the concentrated sample used in the next experimental phase.

5.11. Protein measurement

5.11.1. Introduction

Protein normalisation and measurement is an important procedure. I used the Biuret method using Biorad reagents.

5.11.2. Method in detail

1. A Maxisorp 96 well microtitre plate was used. On each run, one well was left blank. The samples (serum, supernatants etc.) were diluted as appropriate. Standards were run on each plate as follows: 400mg/l, 900 mg/l, 1500 mg/l.
2. Reagent A (Biorad) (25 µl per well) was loaded.
3. The sample was then loaded (10 µl per well) and mixed with gentle agitation.
4. Then, Reagent B was loaded (200 µl per well), and the plate was agitated briefly to mix.
5. The plate was left for 10 minutes.
6. The plate was read using Wallac plate reader at wavelength 750 nm.
7. The standards were plotted, and the values calculated from the standards.

5.12. Purifying specific proteins- immunoglobulin G

5.12.1. Introduction

It is possible to purify immunoglobulin G (IgG) using affinity chromatography. Affinity chromatography uses a reversible interaction between a ligand and the protein of interest. The ligand is irreversibly attached to the column matrix. The ligand then reversibly interacts with the protein of interest, whereas the contaminating molecules flow through the column. The interaction between the protein and ligand is then reversed by altering the column conditions (e.g. changing the pH or salt concentration). In the case of IgG, protein A is the ligand. Protein A is derived from Stapholococcus aureus, and has 5 regions that bind to the Fc part of IgG molecules. Protein A is irreversibly bound to the Sepharose matrix, which allows the protein A molecules access to the IgG as they flow through the column. The column mainly binds IgG (mainly IgG 1, 2 and 4), although IgA and IgM have occasionally been reported to bind.

5.12.2. Protein A chromatography method in detail

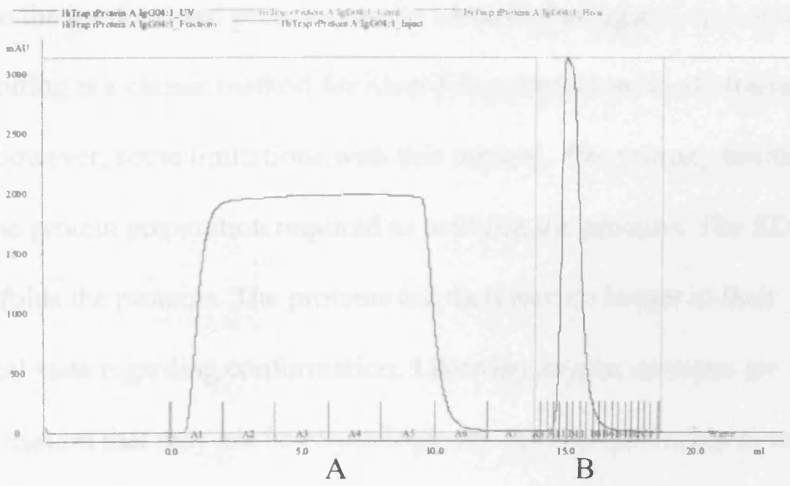
1. The binding buffer (0.02M Sodium Phosphate, pH 7.0) and the elution buffer (0.1M Citric acid, pH 3.0) were made fresh. The column and flow loop were washed thoroughly with 20% ethanol and then washed with binding buffer.
2. The protein A column was connected to the FPLC and bubbles avoided entering the column by dripping binding buffer into the column before attaching to the FPLC. The column was then eluted with 5 column volumes and equilibrated with binding buffer (to remove any residual IgG from previous experiments).

3. The patient serum was then diluted 5-10 fold with binding buffer.
4. The dilute serum was loaded into the flow loop.
5. The FPLC was then programmed as follows:

Flow rate	0.5ml/min
Equilibrate	5 column volumes of binding buffer
Inject	Less than the sample volume
Flow through fraction size	2ml
Re-equilibrate	5 column volumes
Elution fraction size	0.25ml

6. The chromatogram should be as follows:

Figure 5.11. Protein A IgG purification



The sample is injected, and serum constituents other than IgG flow through the column (A). Then during the elution, the IgG is purified (B).

7. The IgG was purified in elution buffer (pH 3.0). The acidity can alter IgG function, and should be normalised with Tris-HCl (pH 9.0). I found that 60µl of Tris-HCl was required to neutralise 250µl of eluted IgG. I ensured the pH

was physiologically normal (pH 7.4) before performing experiments involving live neurones.

Immunology- methods

5.13. Western blotting

5.13.1. Introduction

Western blotting, or Western transfer describes the transfer of proteins from the gel onto an absorbent surface such as nitrocellulose. Once immobilised onto nitrocellulose, the patient serum can be incubated with the nitrocellose. Human IgG that binds to the immobilised proteins can be identified using anti-IgG antibodies. Western blotting is a classic method for identifying protein-antibody interactions. There are, however, some limitations with this method. The primary limitation is related to the protein preparation required to solbilise the proteins. The SDS or LDS solution unfolds the proteins. The proteins are therefore no longer in their physiological state regarding conformation. Likewise, cryptic epitopes are revealed by SDS solubilisation that may not be physiologically seen by antibodies *in vivo*. However, Western blotting provides a valuable method of identifying antibody-antigen interaction.

5.13.2. Method in detail

1. The following transfer buffer was made up: For 500ml of transfer buffer, 25ml of NuPAGE transfer buffer was added to 100ml methanol and 375ml

distilled water. The transfer buffer was mixed and allowed to settle to minimise air bubbles. Then the following were placed in separate trays and incubated with transfer buffer: 5-6 blotting pads, 5-6 filter paper per gel, 1 nitrocellulose paper per gel, cellophane.

2. The gel was processed as for PAGE method. The gel was inserted into the gel membrane sandwich as follows. Blotting pads were laid onto the bottom of the membrane cassette first, followed by at least one filter paper. Next the cassette was laid onto the bench and opened with the lane facing upwards. The gel was then laid onto a filter paper and the gel pushed onto the filter paper. The nitrocellulose paper was then laid onto the other gel surface and any air bubbles were pushed out. The gel should be completely flush with the nitrocellulose paper. The filter paper-gel-nitrocellulose was then loaded into the membrane cassette. Then the cellophane was placed onto the back of the nitrocellulose (to prevent passage of the proteins through the nitrocellulose). Then a further filter paper was added, and the cassette sandwich was completed with blotting pads. The lid was put on the sandwich and I made sure that the sandwich contents were tightly packed.
3. The membrane sandwich was placed into the mini-cell tank and the wedges inserted into the back of the tank to tighten the system and prevent leaks.
4. Next the transfer buffer was poured into the cassette and the tank checked that there were no leaks. The transfer buffer was added until the buffer covers the top of the sandwich contents. Next tap water was added to the surrounding tank as a cooling measure.
5. The tank was then placed onto the platform of the PowerEase 500 and the lid was secured onto the tank.

6. I used the Gel transfer programme that provided 30 Volts with 170 mA current for 1 hr 20 minutes.
7. Once the transfer was complete, the nitrocellulose blot was removed from the sandwich and incubated in 2% milk protein (in 0.9% saline) for 2 hours to block the nitrocellulose. This reduced non-specific interaction of patient serum with the nitrocellulose.
8. The blot was then ready for probing with patient serum. The blot can be washed with 0.9% saline and incubated in shallow trays or inserted into a manifold. Manifolds allow multiple sample or serums to be run alongside one another for comparison.
9. The manifold was washed thoroughly in advance and allowed to dry. The manifold was assembled with the nitrocellulose blot inserted. The manifold was held together with screws and tightened to avoid leakage. The manifold lanes were checked for leaks by loading 0.9% saline into each lane.
10. Samples were diluted and prepared shortly before loading onto the manifold.
11. A clear record of the loading procedure was kept for later analysis. The molecular weight marker was loaded into lane 1, and lane 2 contained the negative control (secondary antibody only). Then the patient's sera were loaded into the appropriate lane as per protocol.
12. The manifold was then incubated overnight at $4^{o}C$ on a rocker.
13. Next the blot was washed before incubation with the secondary antibody. First the blot is washed with 6 changes of tap water. The wash solution was 0.9% saline with 0.2% milk proteins and 0.025% Tween for colormetric development. When using ECL, the wash solution contained 0.05% Tween to reduce the background reactivity.

14. Using this wash solution, the blot was washed every 10 minutes for 10 changes of wash solution.
15. Next the secondary antibody was loaded onto the manifold. The secondary antibody was diluted 1:1000 for colormetric analysis, or 1:5000 for ECL development. The anti-human IgG antibody was conjugated to horseradish peroxidase. The secondary antibody was diluted in 0.2% milk and 0.9% saline. The secondary antibody was incubated for 2 hours.
16. Then the blot was washed exactly as before.
17. At the end of the washing, the blot was ready for development. The manifold was dismantled, and the blot is transferred to a tray. The blot is then developed for 15-20 minutes in the developing solution for colormetric development or using PIERCE enhanced chemiluminscence method (appendix 5).
18. The development was then stopped by washing thoroughly with distilled water, and then dried under a hair drier.
19. The blot was then interpreted.

5.14. Enzyme linked immunosorbent assay

5.14.1. Introduction

Enzyme linked immunosorbent assay (ELISA) offers an alternative way of assessing the presence of antibodies against an antigen. Unlike PAGE with SDS solubilisation, proteins can be immobilised to the ELISA plate in the proteins physiological status and conformation. The proteins are immobilised onto a solid-phase surface. In the case of ELISA, the solid-phase is polystyrene flat-bottomed plates. These plates allow

good optical and binding properties (passively adsorbed). I routinely used Nunc Maxisorp plates.

5.14.2. Method

1. The first variable to be decided was the concentration of the antigen to be used. This varied according to a number of factors, and could only be determined by screening experiments. The protein was pipetted into a vial, and diluted to the desired concentration with 0.05 Molar carbonate buffer. The solution was mixed thoroughly on the vortex, then 100µl of solution was pipetted per well. The plate was then agitated on the ELISA plate shaker for 10 minutes. Then the ELISA plate was left in the cold room (4°C) overnight to allow adsorption of proteins to the plate surface.
2. The wash solution was prepared (saline with 0.2% milk and 0.05% Tween). The plate was washed 6 times with the wash solution, and then blocked with 2% bovine specific albumin (BSA) for 1 hour. The plate was agitated on the ELISA plate shaker during this time.
3. Again the plate was washed 6 times with the wash solution. At this time, the patient samples were prepared in the appropriate dilution. In addition, one well was left blank, and a positive and negative control was run on each plate.
4. 200µl of diluted serum (or control) was loaded into each well. An ELISA template was kept with the details of which patient/control sample was loaded into which well. The plate was incubated at room temperature on the ELISA plate shaker for 1 hour. Samples were usually run in duplicate.
5. Again the plate was washed with 6 changes of wash solution as before.
6. The secondary antibody was then incubated in each well, to allow detection of human IgG that was bound to antigen immobilised on the plate surface. The

secondary antibody for patient and control serum was rabbit anti-human IgG HRP conjugated, at a dilution of 1:1000. 200µl of secondary antibody solution was incubated in each plate. Obviously for commercial antibodies, the correct species of antibody (e.g. goat, mouse) must be chosen. The secondary antibody was incubated for 1 hour.

7. The plate was again washed with wash solution for 6 changes as before.
8. Now the wells were ready to detect the antibody presence. The secondary antibody was conjugated with HRP. The peroxidase catalysed the chemical reaction resulting in colour production in the ELISA well. The detector solution is described in appendix 5 (100µl per well).
9. The plate was allowed to develop in the dark for 15 minutes, and then stopped with 1 M HCl (50 µl per well).
10. The plate was read using the Wallac plate reader. The reading was set for OPD detection at a wavelength of 492nm.
11. The readings were normalised by subtracting the blank, and the duplicates were averaged. The duplicates were compared to ensure reproducibility. If there is poor reproducibility (>10% difference), then the sample was repeated. The same positive and negative controls were run on each plate so that inter-assay comparisons could be made.

5.15. Absorption experiments

5.15.1. Introduction

There are a number of ways to confirm or refute the identity of candidate autoantigens. One method is to perform absorption experiments. These experiments

involve pre-incubating the patient IgG with the candidate antigen (or control) to see if the specific antibodies are removed.

5.15.2. Method in detail

1. 10 µl of patient serum was diluted to 1ml with 0.2% milk in 0.9% normal saline.
2. Next, the antigen to be pre-incubated was added to the serum as required. In addition, the same amount of control antigen was added to a control vial.
3. The vials were secured in a tray and rocked gently at room temperature for 2 hours. Prolonged incubations could also be considered.
4. Next, the vials were centrifuged at 10,000g for 10 minutes.
5. It was important not to disturb any immune complexes or antigen-antibody complexes that may have precipitated. The supernatant (800 µl) was therefore pipetted carefully from the vial. This supernatant was then loaded into a manifold lane to be Western blotted, or alternatively ran on an ELISA (compared with the pre-incubation serum).

Chapter 6. Proteomic identification of the 45 and 98 kDa antigens

6.1. General aim

In patients with post-streptococcal movement and psychiatric disorders, it is possible to demonstrate serum antibodies that bind to a number of apparently common brain autoantigens, as demonstrated by AJ Church. I aimed to identify these autoantigens using protein separation and purification strategies followed by mass spectrometry analysis. A number of different autoantigens have been demonstrated, with varying frequencies. I started with the 98 kDa and subsequently the 45 kDa doublet.

The first aim was to determine whether the autoantigens present in human basal ganglia are also present in rat brain. If this were the case, it would allow me to use brain tissue with a readily available supply, which would improve the chance of identifying the autoantigens.

6.2. Choosing the antigen: Rat brain and human basal ganglia.

6.2.1.The 98kDa autoantigen

6.2.1.1. Method

Human basal ganglia and rat brain (without cerebellum) was homogenised and the supernatant was prepared as previously described. The total protein was measured in the supernatant. The protein was loaded as stated in the table below.

Patients and controls	1 patient with 98 kDa autoantibodies (SN) Control: secondary antibody only
Antigen	Human basal ganglia homogenate 5µg (lane 1) Rat brain homogenate 15µg (lane 2)

Western blotting	Standard method (4-12% Bis-Tris 10 well gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development

6.2.1.2. Results

The 98 kDa autoantigen was present in both human basal ganglia and rat brain (Figure 6.1.). There was secondary antibody reactivity to the human IgG present in the human tissue. The secondary antibody alone demonstrated binding to the human IgG, but not the 98 kDa autoantigen (not shown).

6.2.1.3. Conclusion

The 98kDa autoantigen was present in both human and rat brain tissue. Rat brain will hopefully provide a more readily available source of protein for purification and identification.

6.2.2. The 45 kDa doublet

6.2.2.1. Method

Human basal ganglia and rat brain (without cerebellum) was homogenised and the supernatant was prepared as previously described. The protein was measured in the supernatant and loaded as stated in the table.

Patients and controls	1 patient with 45 kDa autoantibodies (DA) Control: secondary antibody only
Antigen	Human basal ganglia homogenate 5µg (lane 1) Rat brain homogenate 15µg (lane 2)
Western blotting	Standard method (4-12% Bis-Tris 10 well gel)

Serum dilution	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development

6.2.2.2. Results

The 45 kDa autoantigens were present in both human basal ganglia and rat brain (Figure 6.2). There was secondary antibody reactivity to the human IgG present in the human tissue only. The secondary antibody alone demonstrated binding to the human IgG, but not the 45 kDa autoantigen (not shown). These findings were true of both the proteins in the 45 kDa doublet (upper and lower band).

6.3. Comparing antibody findings in different patients

The 45kDa and 98 kDa autoantigens were present in rat, as well as human brain. I next aimed to determine whether different patients with post-streptococcal brain disorders had antibodies against the 45 and 98 kDa autoantigens. I therefore tested different patients with suspected antibodies against the 45 and 98 kDa autoantigens.

6.3.1. The 98kDa autoantigen

6.3.1.1. Method

I therefore tested different patients against rat brain using Western immunoblotting. Rat brain homogenate was diluted to achieve the best signal, without compromising the background. A dilution of 1:16 was found to be appropriate.

Patients and controls	7 patients with suspected 98 kDa autoantibodies (SN, DL, MH, PW, JW, ZB, TT) Control: secondary antibody only
Antigen	1:16 rat brain homogenate 150µl
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300

Antibody detection	Anti-human IgG 1/1000. Colormetric development

6.3.1.2. Result

The 98 kDa autoantibodies were common to different patients with post-streptococcal brain patients (lane 1-6 and 8). The secondary antibody did not react with any rat brain constituents (Lane 7, Figure 6.3.).

6.3.2. The 45kDa doublet autoantigens

6.3.2.1. Method

I tested different patients with suspected 45kDa autoantibodies against rat brain using Western immunoblotting.

Patients and controls	5 patients with one or both of the 45 kDa doublet autoantibodies (TT, GW, DA, EW, Ta) Control: secondary antibody only
Antigen	1:16 rat brain homogenate 150µl
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development

6.3.2.2. Results

A number of post-streptococcal brain patients had antibodies that react with a doublet at 45 kDa. Some patients have both auto-antibodies, some have one only (Figure 6.4.).

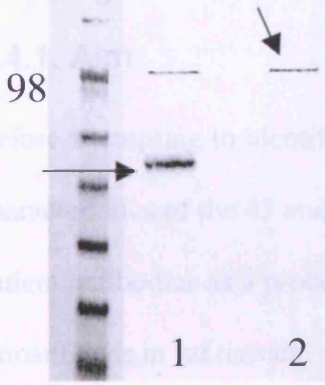


Figure 6.1. Comparison of human basal ganglia with rat brain (98 kDa). Patient SN has antibody reactivity to the 98kDa autoantigen in both human basal ganglia (lane 1) and rat brain (lane 2). Human IgG reactivity (from the secondary antibody) is present in the human tissue only.

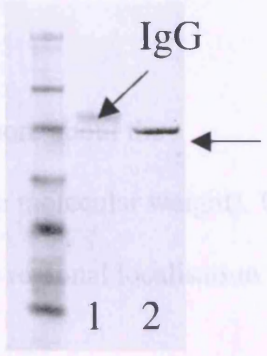


Figure 6.2. Comparison of human basal ganglia (lane 1) with rat brain (lane 2) (45 kDa). Autoantibodies against the 45 kDa autoantigen bind to a 45 kDa autoantigen that is present in both human basal ganglia and rat brain. The secondary antibody binds to human IgG, in the human tissue only.

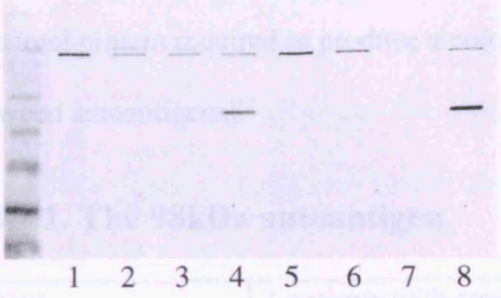


Figure 6.3. Different patients with 98 kDa antigen auto-antibodies. Patients (lanes 1-6 and 8) react with a common 98 kDa autoantigen. Lanes 4 and 8 also have antibodies against a 60 kDa autoantigen. Secondary antibody (lane 7) shows no binding.

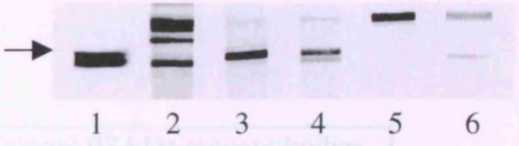


Figure 6.4. Different patients with 45 kDa antigen auto-antibodies. Patients in lanes 1-4 have antibodies that bind to one or both proteins at 45 kDa. Lane 1(both proteins), lane 2(lower protein), lanes 3 and 4 (both), lane 6 (lower protein). Lanes 2-6 also appear to have a common antibody to a 60 kDa protein.

6.4. Regional distribution of the 45 kDa and 98 kDa proteins.

6.4.1. Aim

Before attempting to identify the proteins, I tried to learn more about the characteristics of the 45 and 98 kDa proteins (other than the molecular weight). Using patient antibodies as a probe, I next aimed to determine the regional localisation of the autoantigens in rat tissues.

6.4.2. Method

The rat organs were from male Wistar rats. The organs were removed immediately after death, and snap frozen. The organs were kept whole at –80 °C until use, then homogenised and centrifuged as previously detailed. The supernatant was stored in alloquots at –80°C until required. In order to make comparisons between tissues, I measured the total protein (Biuret method, as previously detailed) for all tissues. The total proteins were then normalised and loaded into appropriate lanes in a 10 well gel. The total protein required to produce a colormetric signal is stated below (and varied between autoantigens).

6.4.2.1. The 98kDa autoantigen

Patient	1 patients with strong signal 98 kDa autoantibodies (SN)
Antigen	Lane 1. Rat brain (cerebellum removed) 6.25µg. Lane 2. Rat cerebellum 6.25µg. Lane 3. Rat kidney 6.25µg. Lane 4. Rat liver 6.25µg. Lane 5. Rat heart 6.25µg.
Western blotting	Standard method (4-12% Bis-Tris 10 well gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development

6.4.2.2. The 45kDa doublet

Patient	1 patients with strong signal 45 kDa doublet autoantibodies (EW)
Antigen	Lane 1. Rat brain (cerebellum removed) 25µg. Lane 2. Rat cerebellum 25µg. Lane 3. Rat kidney 25µg. Lane 4. Rat liver 25µg. Lane 5. Rat heart 25µg.
Western blotting	Standard method (4-12% Bis-Tris 10 well gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development

As can be seen, the total proteins needed to be higher using this patient antibody and the 45 kDa antigens.

6.4.3. Results

6.4.3.1. The 98 kDa protein

Using the auto-antibodies as a probe, it was possible to establish the regional localisation of the 98kDa protein. The protein was expressed in rat brain and weakly in rat heart. There is no apparent difference between whole brain (without cerebellum) and cerebellum (Figure 6.5).

6.4.3.2. The 45 kDa doublet

Using the auto-antibodies as a probe, it was possible to establish the regional localisation of the 45 kDa doublet proteins. The lower protein is present in whole brain and cerebellum, but not in other organs. By contrast, the upper protein in the doublet is ubiquitous, and appears to be equally present in all tissues (Figure 6.6.).

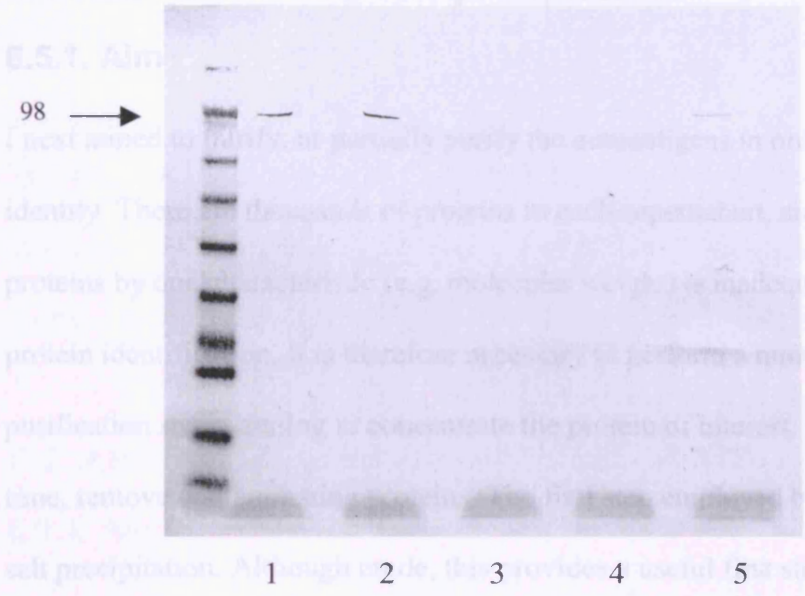


Figure 6.5. Regional comparison between brain and other organs. The 98 kDa autoantigen is highly expressed in brain (whole brain, lane 1 and cerebellum, lane 2) and weakly in heart (lane 5), but not in rat kidney and liver (lanes 3 and 4).

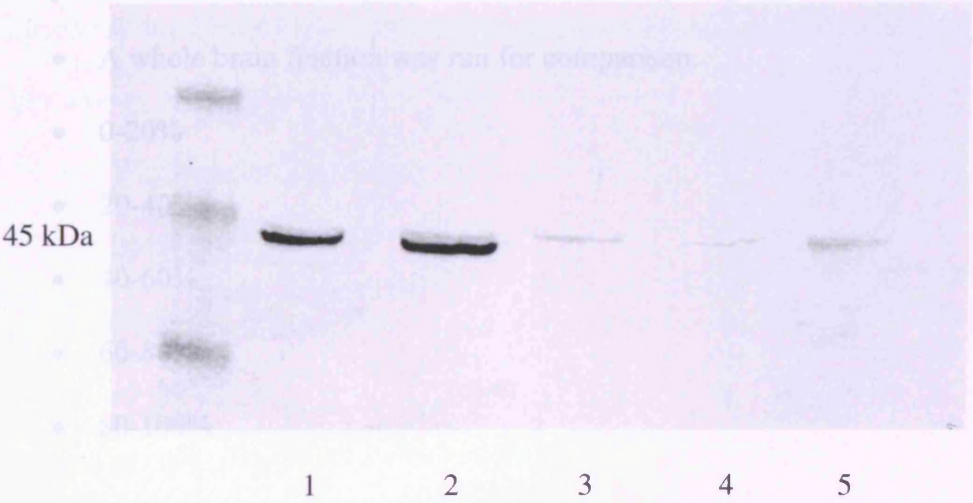


Figure 6.6. Regional comparison between brain and other organs (45 kDa doublet). The lower protein is present only in whole brain and cerebellum (lanes 1 and 2). By contrast, the upper 45kDa protein is ubiquitous, and equally present in all tissues.

6.5. Ammonium sulphate precipitation

6.5.1. Aim

I next aimed to purify, or partially purify the autoantigens in order to determine their identity. There are thousands of proteins in each supernatant, and separating the proteins by one characteristic (e.g. molecular weight) is inadequate for confident protein identification. It is therefore necessary to perform a number of different purification steps, aiming to concentrate the protein of interest, whilst at the same time, remove contaminating proteins. The first step employed by investigators is often salt precipitation. Although crude, this provides a useful first step.

6.5.2. Method

The method has been previously described in detail. Again the patient IgG is used as the probe. I initially used large ammonium sulphate precipitation fractions as follows:

- A whole brain fraction was run for comparison.
- 0-20%
- 20-40%
- 40-60%
- 60-80%
- 80-100%

The precipitated proteins were re-suspended in 1ml of sodium phosphate buffer pH 7.0, and mixed on the vortex. For this experiment the proteins were not desalted. The different fractions were then loaded onto a 10 well 4-12% Bis-Tris gel, electrophoresed using the standard method and transferred onto nitrocellulose using

the standard method. The blot was blocked using 2% milk for 2 hours. Patient SN was used to determine which fraction contained the 98 kDa protein, DA was used for the 45kDa proteins.

6.5.3. Results

The 98 kDa protein precipitates mostly between 60-80% salt fractionation, although also precipitates to a lesser extent in the 40-60% fraction (Figure 6.7.). The 45 kDa proteins also precipitated in the 40-60% and 60-80% fraction.

6.6. Ammonium sulphate precipitation with smaller fractions

6.6.1. Aim

I next aimed to see whether I could improve separation further by reducing the size of the fractions, although the yield of protein decreased as a consequence.

6.6.2. Method

I used the same ammonium sulphate precipitation method, but this time used smaller fractions as follows:

- Whole brain as a control
- 50-60%
- 60-65%
- 65-70%
- 70-75%
- 75-80%
- 80-85%
- 85-90%
- 90-100%

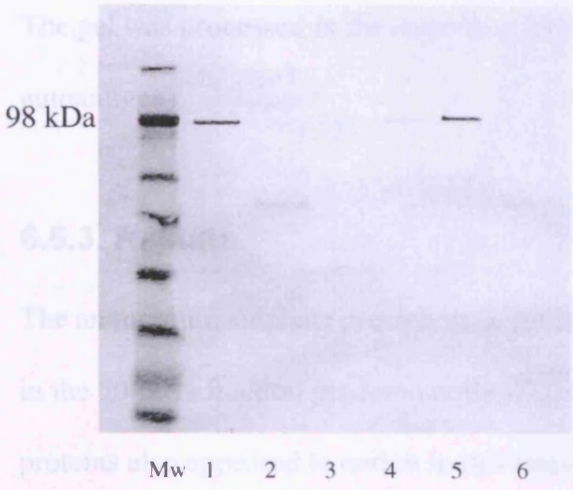


Figure 6.7. Ammonium sulphate precipitation. Fractions electrophoresed and blotted onto nitrocellulose (1: whole. 2: 0-20%. 3: 20-40%. 4: 40-60%. 5:60-80%. 6: 80-100%). Patient SN with 98 kDa antibodies used to probe the blot. The 98kDa protein precipitates mainly in the 60-80% fraction, although to a lesser extent in the 40-60% fraction.

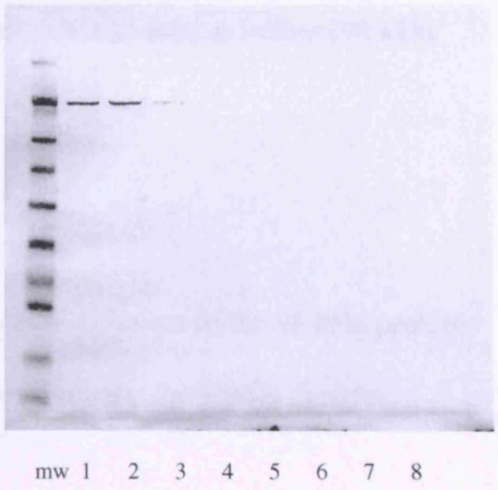


Figure 6.8. Second ammonium sulphate precipitation screen. Whole fraction is in lane 1. The 98 kDa protein is enriched in the 50-60% fracion (lane 2), and to a lesser extent 60-65% fraction (lane 3).

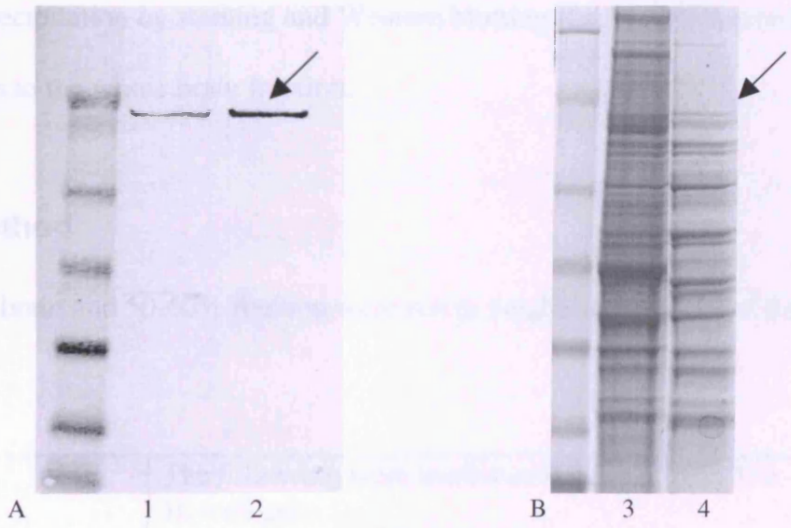


Figure 6.9. Result of ammonium sulphate precipiation. Western blotting (A) of whole rat brain (lane 1) and 50-60% (lane 2). Patient SN with 98 kDa antibodies. Silver staining of whole rat brain (B) (lane 3) and 50-60% fraction (lane 4). The silver stain demonstrates a reduction in the background contamination.

The gel was processed in the same way, and patient SN was used as before (98 kDa autoantigen).

6.6.3. Results

The ammonium sulphate precipitation demonstrated enrichment of the 98 kDa protein in the 50-60% fraction predominantly (Figure 6.8.). Notably, the 45kDa doublet proteins also appeared to enrich in this fraction.

6.7. Ammonium sulphate precipitation final fraction

6.7.1. Aim

Finally, I aimed to crudely examine the degree of purification achieved by ammonium sulphate precipitation by staining and Western blotting the 50-60% fraction, with comparison to the whole brain fraction.

6.7.2. Method

The whole brain and 50-60% fraction were run in neighbouring lanes as follows:

Antigen	The following were loaded onto a 4-12% Bis-Tris 10 well gel: Lane 1. Rat whole brain 6.25µg. Lane 2. Rat 50-60% fraction 6.25µg. Lane 3. Rat whole brain 6.25µg. Lane 4. Rat 50-60% fraction 6.25µg.
Silver staining	Lanes 3 and 4 were silver stained using the routine method
Western blotting	Lanes 1 and 2 were used for Western blotting using

	the standard method
Serum dilution for Western blotting	1/300
Antibody detection for Western blotting	Anti-human IgG 1/1000. Colormetric development

6.7.3. Results

The 98 kDa protein was confirmed to reside in the 50-60% fraction using Western blotting. The silver stain showed that there had been a significant reduction in contamination. The 98kDa protein now appeared to be more discrete, although further purification will be necessary (Figure 6.9.). A similar degree of purification was evident in the 45 kDa doublet proteins.

6.8. 2-dimensional electrophoresis

6.8.1. Aim

The first purification step (ammonium sulphate precipitation) allowed partial purification of the protein of interest. This improved the chances of adequate separation of proteins. Next I used 2-dimensional electrophoresis to separate the proteins. This method allowed proteins to be separated according to mass and charge. If this method achieved adequate separation, the candidate protein would be cut out of the gel and subjected to mass spectrometry.

6.8.2. Method

6.8.2.1. The 98 kDa protein

The method is previously described in detail in chapter 5.

Antigen	50-60% fraction, desalted to sodium phosphate buffer pH 7.0. 10µg loaded per isoelectric focussing lane.
Isoelectric focussing	Standard protocol as previously described.
2nd stage: PAGE electrophoresis	Standard protocol as previously described. MES buffer.
Western blotting of gel 1	Standard method.
Western blotting patient antibody	SN 1:300 dilution
Western blotting secondary antibody	Rabbit anti-human IgG HRP conjugated 1:1000
Western blotting development	Colormetric and ECL.
Silver staining of gel 2	Standard silver staining method.

6.8.2.2. The 45 kDa doublet

Antigen	50-60% fraction, desalted to sodium phosphate buffer pH 7.0. 10µg loaded per isoelectric focussing lane.
Isoelectric focussing	Standard protocol as previously described.
2nd stage: PAGE electrophoresis	Standard protocol as previously described. MES buffer.
Western blotting of gel 1	Standard method.
Western blotting patient antibody	DA 1:300 dilution (upper 45 kDa band) EW 1:300 dilution (both 45 kDa bands)
Western blotting secondary antibody	Rabbit anti-human IgG HRP conjugated 1:1000
Western blotting development	Colormetric and ECL.
Silver staining of gel 2	Standard method.

6.8.3. Results

6.8.3.1.The 98 kDa autoantigen

Initial attempts were unsuccessful related to the following problems and probable causes:

Problem	Solution
Very streaky staining pattern	Ensure salt at 20mMol or below (sodium phosphate buffer desalting)

Poor resolution of proteins with streaking	Reduced protein load to 10μg per IEF lane.
Poor visualisation on Western blotting	Conversion to ECL development (and reduction of secondary antibody to 1:5000. Increase tween concentration to 0.1% to reduce background.
Correlation between silver stained and Western blotting gels	Ensure the IEF lanes are trimmed exactly the same way before loading onto the PAGE 2D gel for the 2nd stage.

After correcting for the above problems, it enabled me to achieve reasonable separation of proteins and allow the candidate protein to be identified on Western blotting. As can be seen from Figure 6.10, there were 2 possible proteins apparent on the silver stained gel. I therefore subjected both proteins to mass spectrometry. I was able to reproduce the Western blots with satisfactory results using a different patient (DL).

6.8.3.2. The 45 kDa doublet autoantigens

Patient DA with the upper doublet 45 kDa protein bound to a protein more basic than the 98 kDa NSE. The upper 45 kDa protein had a pI of approximately 6 (Figure 6.11.). The lower 45 kDa protein had a similar pI to the 98 kDa protein. Both the upper and lower 45 kDa protein could be localised on the silver stained gels (Figure 6.11. and 6.12).

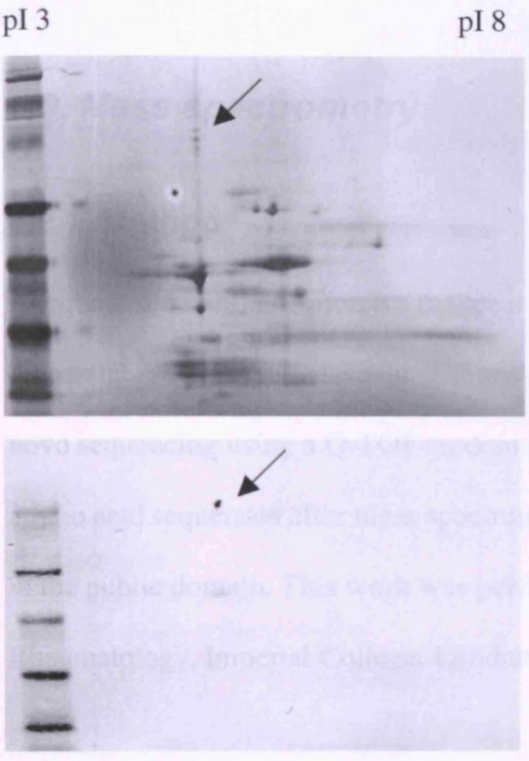


Figure 6.10. 2-dimensional electrophoresis. The silver stain allowed adequate separation of proteins (upper figure). Western blotting (lower figure) allowed identification of the 98 kDa autoantigen using patient SN IgG.

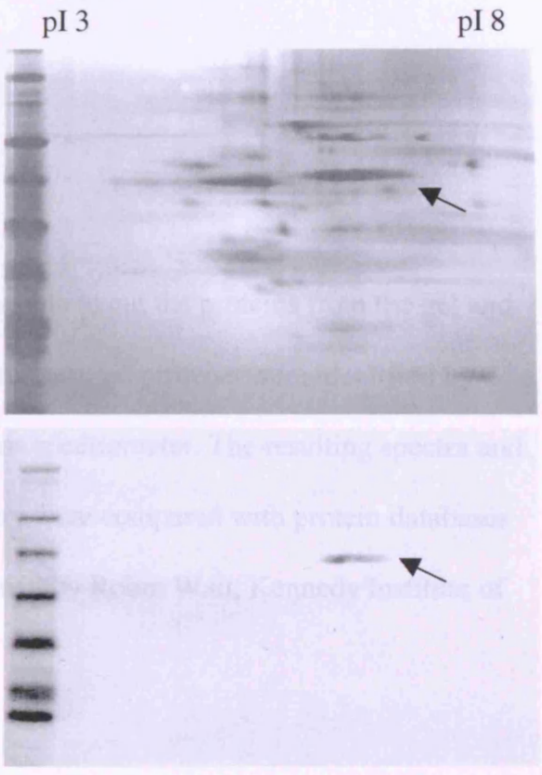


Figure 6.11. 2-dimensional electrophoresis using DA serum (upper doublet of 45 kDa autoantigen). The suspected protein of interest is labelled with an arrow.

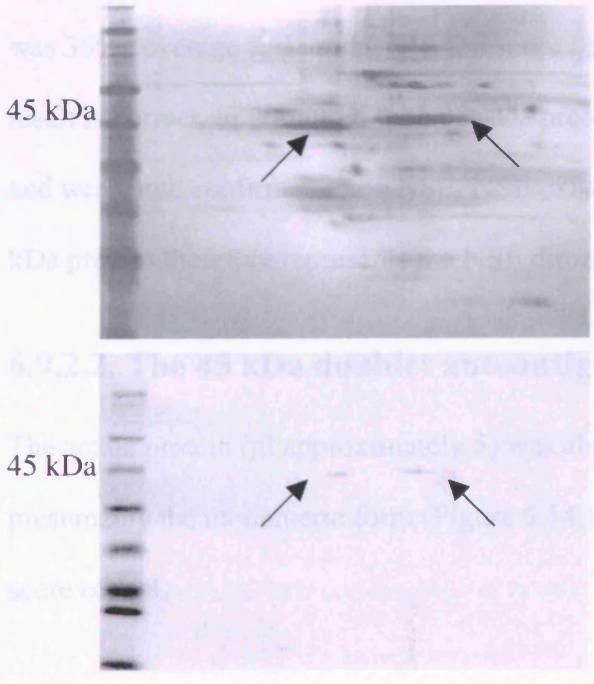


Figure 6.12. Western blotting (lower image) using patient EW showing binding to the more basic upper protein, and the more acidic lower protein.

6.9. Mass spectrometry

6.9.1. Method

Adequate separation of proteins makes it possible to cut the proteins from the gel and digest the proteins using trypsin. The resulting purified proteins were identified by de novo sequencing using a Q-TOF tandem mass spectrometer. The resulting spectra and amino acid sequences after mass spectrometry were compared with protein databases in the public domain. This work was performed by Robin Wait, Kennedy Institute of Rheumatology, Imperial College, London.

6.9.2. Results

6.9.2.1. The 98 kDa autoantigen

As can be seen, a large number of amino acid sequences in the 98 kDa protein were compatible with gamma enolase, neuron-specific enolase (NSE) (Figure 6.13). There was 36% coverage and produced a lod score of 930 with high confidence that this result is correct. Importantly, both 98 kDa proteins were digested, analysed separately and were both confirmed to be NSE. NSE exists as a monomer or a dimer. The 98 kDa protein therefore represents the NSE dimer.

6.9.2.2. The 45 kDa doublet autoantigens

The acidic protein (pI approximately 5) was also found to be gamma enolase (NSE), presumably the monomeric form (Figure 6.14.). The coverage was 5.3% with a lod score of 198.

ENOG_RAT
Gamma enolase (EC 4.2.1.11) (2-phospho-D-glyc

(P07323)

```
  1      SIQKIWAREI LDSRGNPTVE VDLHTAKGLF RAAVPSGAST GIYEALELRD GDKQRYLGKG VLKAVDHINS
 71      TIAPALISSG LSVVEQEKLD NLMLELDGTE NKSKFGANAI LGVSLAVCKA GAAEKDLPLY RHIAQLAGNS
141      DLILPVPAFN VINGGSHAGN KLAMQEFMIL PVGAESFRDA MRLGAEVYHT LKGVIKDKYG KDATNVGDEG
211      GFAPNILENS EALELVKEAI DKAGYTEKMV IGMDVAASEF YRDGKYDLDF KSPADPSRCI TGDQLGALYQ
281      DFVRNYPVVS IEDPFDQDDW AAWSKFTANV GIQIVGDDLT VTNPKRIERA VEEKACNCLL LKVNQIGSVT
351      EAIQACKLAQ ENGWGVMVSH RSGETEDTFI ADLVVGLCTG QIKTGAPCRS ERLAKYNQLM RIEEELGEEA
421      RFAGHNFRNP SVL
```

Figure 6.13. Mass spectrometry result of 98 kDa protein.

ENOG_RAT
Gamma enolase (EC 4.2.1.11) (2-phospho-D-glyc

(P07323)

```
  1      SIQKIWAREI LDSRGNPTVE VDLHTAKGLF RAAVPSGAST GIYEALELRD GDKQRYLGKG VLKAVDHINS
 71      TIAPALISSG LSVVEQEKLD NLMLELDGTE NKSKFGANAI LGVSLAVCKA GAAEKDLPLY RHIAQLAGNS
141      DLILPVPAFN VINGGSHAGN KLAMQEFMIL PVGAESFRDA MRLGAEVYHT LKGVIKDKYG KDATNVGDEG
211      GFAPNILENS EALELVKEAI DKAGYTEKMV IGMDVAASEF YRDGKYDLDF KSPADPSRCI TGDQLGALYQ
281      DFVRNYPVVS IEDPFDQDDW AAWSKFTANV GIQIVGDDLT VTNFKRIERA VEEKACNCLL LKVNQIGSVT
351      EAIQACKLAQ ENGWGVMVSH RSGETEDTFI ADLVVGLCTG QIKTGAPCRS ERLAKYNQLM RIEEELGEEA
421      RFAGHNFRNP SVL
```

Figure 6.14. Mass spectrometry result of 45 kDa lower molecular weight protein.

ENOA_RAT
Alpha enolase (EC 4.2.1.11) (2-phospho-D-glyc

(P04764)

```
  1      SILKIHAREI FDSRGNPTVE VDLYTAKGLF RAAVPSGAST GIYEALELRD NDKTRFMGKG VSKAVEHINK
 71      TIAPALVSKK LNVVEQEKID QLMIEMDGTE NKSKFGANAI LGVSLAVCKA GAVEKGVPLY RHIADLAGNP
141      EVILPVPAFD VINGGSHAGN KLAMQEFMIL PVGASSFREA MRIGAEVYHN LKNVIKEKYG KDATNVGDEG
211      GFAPNILENK EALELLKSAI AKAGYTDQVV IGMDVAASEF YRAGKYDLDF KSPDDASRYI TPDQLADLYK
281      SFIKDYPVVS IEDPFDQDDW DAWQKFTATA GIQVVGDDLT VTNPKRIAKA AGEKSCNCLL LKVNQIGSVT
351      ESLQACKLAQ SNGWGVMVSH RSEETEDTFI ADLVVGLCTG QIKTGAPCRS ERLAKYNQIL RIEEELGSKA
421      KFAGRSFRNP LAK
```

Figure 6.15. Mass spectrometry result of 45 kDa upper molecular weight protein.

The basic protein (pI approximately 6) was found to be alpha enolase (non-neuronal enolase) (Figure 6.15.). The coverage was 9% with a lod score of 383.

6.10. Conclusion of protein purification

The recognised properties of the monomeric and dimeric neuron-specific enolase (NSE), and human non-neuronal enolase (NNE) are presented below.

Autoantigen	Molecular weight, Da	PI	Regional distribution
45 kDa upper	47,051	6.1	Ubiquitous
45 kDa lower	46,991	5.1	Neuronal specific
98 kDa	~87,000	5.1	Neuronal specific

These properties are compatible with the regional distribution of the 98 kDa and 45 Kda doublet autoantigens.

Enolase is a glycolytic enzyme, but is a multifunctional protein. Interestingly, both neuronal and non-neuronal enolase exist on the cell surface of neurones (Lim L et al., 1983; Leung TK et al., 1987), and are involved in cell signalling and energy metabolism (Nakajima K et al., 1994a; Nakajima K et al., 1994b). Also, the glycolytic enzyme enolase exists on the Streptococcal cell surface, and has been previously incriminated in the post-streptococcal autoimmune disease, rheumatic fever (Fontan PA et al., 2000). The enolases would therefore make quite appealing candidate autoantigens.

Next, I planned to confirm these conclusions by using commercial antibodies against the enolases, and commercial enolase antigens.

6.11. Testing commercial enolase antigens

6.11.1. Aim

The candidate autoantigens are neuronal and non-neuronal enolases. I next tested these findings by buying purified human neuronal and non-neuronal enolases from commercial companies.

6.11.2. Method

6.11.2.1. Non-neuronal enolase

Human non-neuronal enolase (NNE) purified from brain was purchased from a commercial source (Accurate chemical and scientific corporation, Westbury, New York). The human protein was purified using chromatography (ion exchange and gel filtration) and was claimed to be at least 96% pure. The human NNE and rat brain were loaded as follows:

Patients and controls	1 patient with anti-NNE antibodies (DA) Control: secondary antibody only
Antigen	Rat brain homogenate 1:16 (lane 1) Human NNE 2 μg (lane 2) Rat brain homogenate 1:16 (lane 3) Human NNE 2 μg (lane 4)
Western blotting	Standard method for lanes 3 and 4 (4-12% Bis-Tris 10 well gel)
Serum dilution for Western blotting	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development
Silver staining	Lanes 1 and 2 were stained using the silver stain method

6.11.2.2. Neuronal specific enolase

Human neuronal specific enolase (NSE) was purchased from a commercial source (appendix). The human protein was purified using chromatography (ion exchange and gel filtration) and was claimed to be at least 96% pure. The human NSE and rat brain were loaded as follows:

Patients and controls	1 patient with anti-NSE antibodies (GW) Control: secondary antibody only
Antigen	Rat brain homogenate 1:16 (lane 1) Human NSE 2 µg (lane 2) Rat brain homogenate 1:16 (lane 3) Human NSE 2 µg (lane 4)
Western blotting	Standard method for lanes 3 and 4 (4-12% Bis-Tris 10 well gel)
Serum dilution for Western blotting	1/300
Antibody detection	Anti-human IgG 1/5000. ECL development
Silver staining	Lanes 1 and 2 were stained using the silver stain method

6.11.3. Results

6.11.3.1. Non-neuronal enolase

Using Western blotting, patient DA reacted with the 45 kDa protein in the whole rat brain preparation (lane 3). The patient reacted with the commercial human NNE (lane 4). The 2 reactive bands lined up on the Western blot (Figure 6.16.). The silver stained gel confirmed the relative purity of the commercial human NNE.

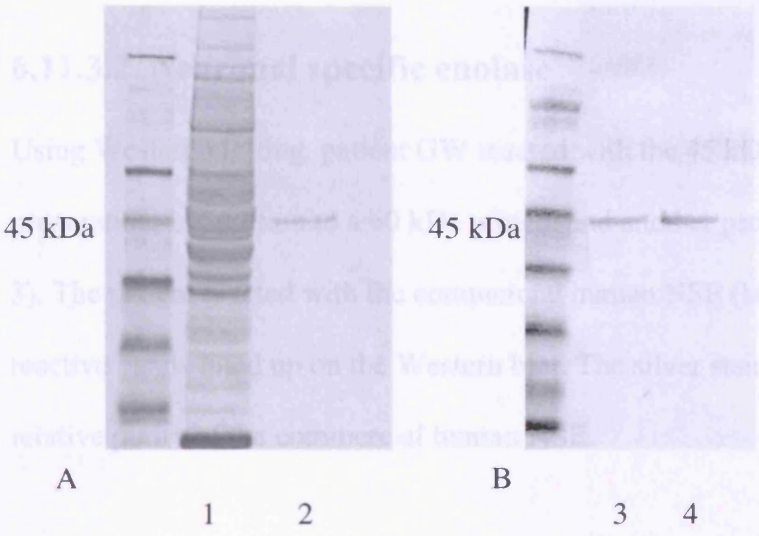


Figure 6.16. Confirmation of anti-NNE antibodies using commercial antigen. Silver staining (A) and Western blot (B) demonstrating that patient DA has anti-NNE antibodies.

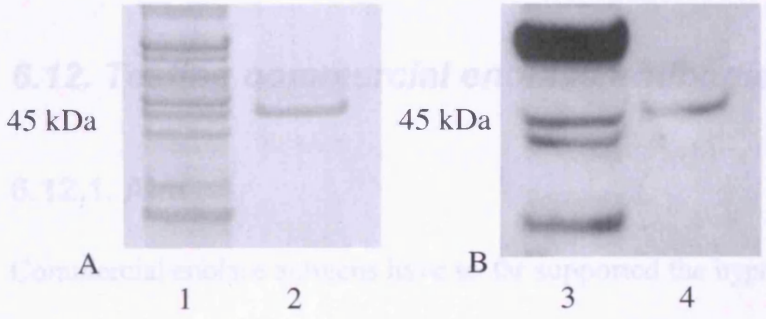


Figure 6.17. Confirmation of anti-NSE antibodies using commercial antigen. Silver staining (A) and Western blot (B) demonstrating that patient GW has anti-NSE antibodies.

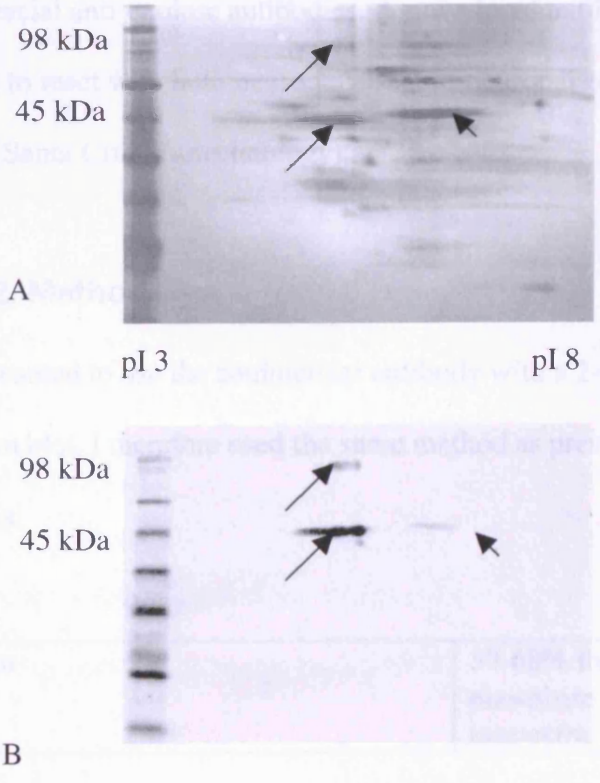


Figure 6.18. 2-dimensional electrophoresis using commercial anti-enolase antibody (C-19) at 1:2000 dilution with colormetric development shows binding to the monomeric and dimeric NSE (long arrows) and NNE (short arrow).

6.11.3.2. Neuronal specific enolase

Using Western blotting, patient GW reacted with the 45 kDa protein in the rat preparation, in addition to a 60 kDa protein and another protein below 45 kDa (lane 3). The patient reacted with the commercial human NSE (lane 4) (Figure 6.17). The 2 reactive bands lined up on the Western blot. The silver stained gel confirmed the relative purity of the commercial human NSE.

6.12. Testing commercial enolase antibodies

6.12.1. Aim

Commercial enolase antigens have so far supported the hypothesis that the 45 kDa antigens are human enolase isoforms. I next aimed to test this hypothesis with commercial anti-enolase antibodies. A polyclonal antibody was used which was known to react with both neuronal and non-neuronal enolases of human and rat origin (C-19, Santa Cruz Biotechnology).

6.12.2. Method

I first wanted to use the commercial antibody with a 2-dimensional electrophoresis Western blot. I therefore used the same method as previously described during patient analysis:

Antigen	50-60% fraction, desalted to sodium phosphate buffer pH 7.0. 10µg loaded per isoelectric focussing lane.

Isoelectric focussing	Standard protocol as previously described.
2nd stage: PAGE electrophoresis	Standard protocol as previously described. MES buffer.
Western blotting of gel 1	Standard method.
Western blotting commercial antibody	Goat C-19 1:2000 dilution
Western blotting secondary antibody	Rabbit anti-goat IgG HRP conjugated 1:1000
Western blotting development	Colormetric.
Silver staining of gel 2	Standard silver staining method.

6.12.3. Results

The commercial antibody was overloaded when originally used at a 1:500 dilution. Even at 1:2000 dilution, a strong signal was present with colormetric development. The C-19 antibody showed binding to the more basic non-neuronal enolase, and to the more acidic neuronal specific enolase in the monomeric and dimeric form as presented in Figure 6.18.

6.13. Testing both commercial anti-enolase antibody and enolase antigens

6.13.1. Aim

I now intended to test a number of patients who had suspected anti-NNE antibodies against the human NNE, using the commercial anti-enolase antibody as a control.

6.13.2. Method

I used commercial human NNE as the antigen, which was loaded onto a 2D gel and electrophoresed using the standard method.

Patients and controls	4 human patients with suspected anti-NNE antibodies (DA, LD,CR,EW) 2 human healthy controls (AC49, AC50) Control: secondary antibody only Positive control: C-19 anti-enolase antibody
Antigen	Human NNE 4 μg
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution for Western blotting	1/300 in human patients and controls 1/2000 for C-19
Antibody detection	Anti-human IgG 1/5000. Anti-goat IgG 1/5000 for C-19 antibody. ECL development

6.13.3. Results

The post-streptococcal neuropsychiatric patients with suspected anti-NNE antibodies all reacted with the commercial NNE. The commercial anti-enolase antibody (C-19) reacted with enolase at the same level of the patients. Neither of the human controls reacted with the antigen (Figure 6.19.). The blot showed some background reactivity, possibly related to inadequate washing. Unfortunately, the human commercial enolase was prohibitive in cost, and alternative NNE sources will be necessary for further experiments.

6.14. Presence of anti-enolase antibodies in CSF, as well as serum.

6.14.1. Aim

The presence of antibodies reactive with brain proteins in the serum is suggestive that a brain disease may be autoantibody mediated. However, presence of these antibodies in the CSF would be a significantly more persuasive argument. I therefore used the CSF of one patient (EW) who had anti-NNE antibodies in the serum to examine for CSF anti-NNE antibodies.

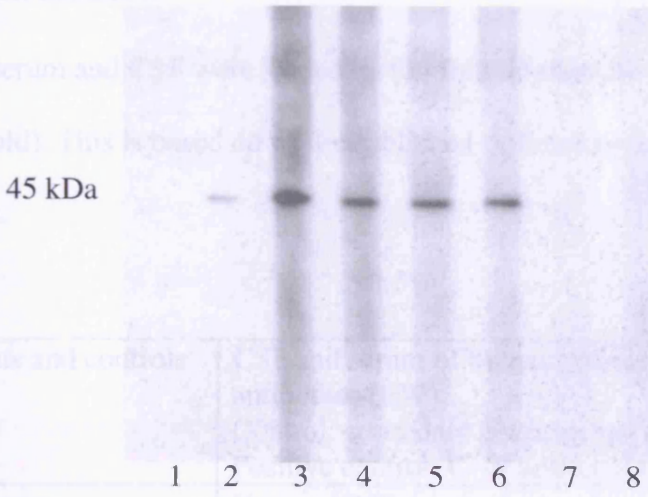


Figure 6.19. Western blotting against commercial human NNE. Secondary antibody only (lane 1) shows no reactivity. Positive control (commercial anti-enolase antibody) reacts with human NNE (lane 2). Human patients all react with the human NNE (lanes 3-6). Human controls (lane 7 and 8) do not show any antibody reactivity.

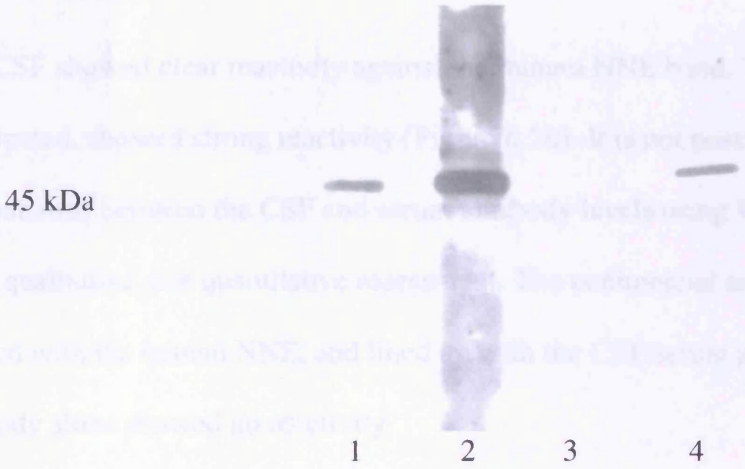


Figure 6.20. Comparison of anti-NNE antibodies in CSF and serum. CSF 1:10 diltution (lane 1) is compared with serum 1:4000 dilution (lane 2). Secondary antibody only (lane 3) shows no reactivity. Commercial anti-enolase antibody (lane 4) shows reactivity with human NNE as anticipated.

6.14.2. Method

The serum and CSF were loaded in 400 fold dilution for comparison (serum diluted 400 fold). This is based on well-established differences in total IgG between CSF and serum.

Patients and controls	CSF and serum of human patient with anti-NNE antibodies (EW) Control: secondary antibody only Positive control: C-19 anti-enolase antibody
Antigen	Human NNE 4 μg
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Dilution for Western blotting	1/10 CSF dilution (200μl in 2ml) 1/4000 serum dilution 1/4000 for C-19
Antibody detection	Anti-human IgG 1/5000. Anti-Goat IgG 1/5000 for C-19 antibody. ECL development

6.14.3. Results

The CSF showed clear reactivity against the human NNE band. The serum, as anticipated, showed strong reactivity (Figure 6.20). It is not possible to make comparisons between the CSF and serum antibody levels using Western blotting due to its qualitative, not quantitative assessment. The commercial anti-enolase antibody reacted with the human NNE, and lined up with the CSF/serum pair. The secondary antibody alone showed no reactivity.

6.15. Anti-NNE antibody ELISA: A cheap available autoantigen source?

6.15.1. Aim

The protein NNE is a relatively conserved protein with relative homology between species. Human NNE is available by purifying the protein from human tissue but is expensive to buy and labour intensive to do, with poor yield of the final protein. Recombinant human enolase is one alternative source (discussed later). However, there are 2 commercially available enolase antigens available that are cheap (Baker's yeast enolase and rabbit muscle enolase).

I tried to determine whether these commercially available enolase proteins would be valid surrogate autoantigens of human NNE.

6.15.2. Method

Using NCBI Protein FASTA technology and BLAST 2 sequence homology analysis, I first compared the sequence homology between human NNE and the 2 commercially available enolase antigens.

Commercial antigen	Homology with human NNE			
	Identities	Positives	Gaps	Chance homology
Baker's yeast enolase	62%	78%	0%	10^{-154}
Rabbit muscle enolase	83%	90%	0%	0.00

As can be seen, there is a high degree of homology between the human NNE and these commercial enolases. It is also conceivable that homology is conformational, rather than the linear peptide sequence.

Next, I aimed to determine whether two patients previously identified with anti-human/rat NNE antibodies reacted with these commercial enolase antigens.

Patients and controls	2 patients with anti-human NNE Abs (DA, EW) Control: Commercial C-19 anti-enolase Ab
Antigen	Human basal ganglia 5μg homogenate (lane 1) Rabbit muscle enolase 5μg (lane 2) Baker's yeast enolase 5μg (lane 3)
Western blotting	Standard method (4-12% Bis-Tris 10 well gel)
Serum dilution	1/300 (human patients) 1/1000 (C-19)
Antibody detection	Anti-human IgG 1/1000. Anti-Goat IgG 1/1000 for C-19 antibody Colormetric development

6.15.3. Result

One gel was stained with colloidal blue staining. Rabbit enolase had one strong band at 45 kDa. Baker's yeast enolase had one strong band at 43 kDa. There were 2 weaker higher bands in both preparations.

C-19 commercial polyclonal anti-enolase antibodies reacted very strongly with all 3 lanes. DA reacted strongly with all 3 antigens (rabbit>Baker's>human basal ganglia)

EW reacted weakly with all 3 antigens (human basal ganglia>rabbit>Baker's).

6.15.4. Conclusion

There was some evidence that the patients have antibodies that react with Rabbit muscle enolase and Baker's yeast enolase, as well as human alpha enolase. However, this did not mean that the anti-human alpha enolase antibodies cross-react with the commercial enolase antigens. Even if the antibodies do cross-react, the antibodies may not have the same affinity to human as they do to other enolases.

I therefore aimed to determine whether the anti-human alpha enolase antibodies do cross-react with the commercial enolase antigens using absorption experiments.

6.16. Cross-reactivity of anti-NNE antibodies with commercial enolase antigens: absorption experiments.

6.16.1. Hypothesis and aim

The next aim was to find a useful, cheap surrogate enolase for ELISA development. Rabbit and Baker's yeast enolase appear to be useful candidates. It was now necessary to determine whether the patient anti-NNE antibodies truly cross-react with the commercial enolase antigens. I therefore performed an absorption experiment, to see if anti-NNE antibodies were removed by pre-incubation with the commercial antigens.

6.16.2. Method

The absorption experiment method is previously described in detail. The following tubes were created. Tube A was a control with no antigen incubation. Serum from 1 patient (DA) was used.

Constituents	Tube A	Tube B	Tube C
Serum	10µl in 1ml 0.2% milk	10µl in 1ml 0.2% milk	10µl in 1ml 0.2% milk
Antigen incubation	-	20µg Rabbit muscle enolase	20µg Baker's yeast enolase
Incubation	2 hours on rocker at room temperature		
Centrifugation	10,000g for 10 minutes		
Sample for incubation with rat brain homogenate	800µl supernatant (do not disturb any precipitate)		

The post-incubation sera were incubated with the rat brain homogenate after Western immunoblotting (standard method). C-19 was used as positive control.

6.16.3. Results

The anti-rat NNE antibody signal intensity was significantly reduced by pre-incubation with rabbit muscle enolase, but not with Baker's yeast enolase. There was still some residual antibody reactivity even with rabbit enolase (Figure 6.21.). A higher amount of rabbit enolase incubation (100 μg) produced no significant difference.

6.16.4. Conclusion

This suggests that the anti-NNE antibodies do cross-react with rabbit muscle enolase in this patient (DA). Rabbit muscle enolase may therefore provide a useful surrogate enolase source for ELISA development. There are still some residual anti-brain enolase antibodies. This may be because the incubated enolase antigen was not adequately in excess, or that some of the anti-NNE antibodies are specific to rat/human enolase epitopes, and do not cross-react with the commercial rabbit muscle enolase preparations. I therefore used rabbit muscle enolase for further ELISA development and assessment with other patients.

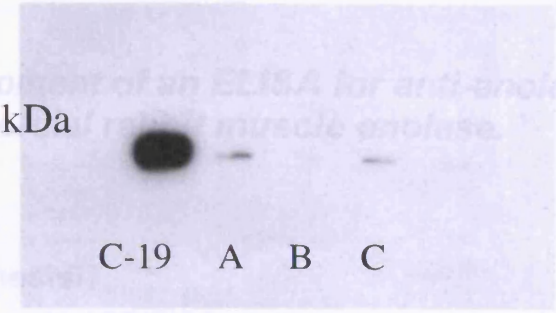


Figure 6.21. Incubation experiment with commercial enolases. Anti-enolase antibody (C-19) binds to alpha enolase in brain homogenate. Patient DA serum used in all incubation experiments. No antigen incubation (lane A). Pre-incubation with rabbit muscle enolase resulted in decreased anti-brain enolase antibodies (lane B). Pre-incubation with Baker's yeast enolase did not bind the anti-brain enolase antibodies (lane C).

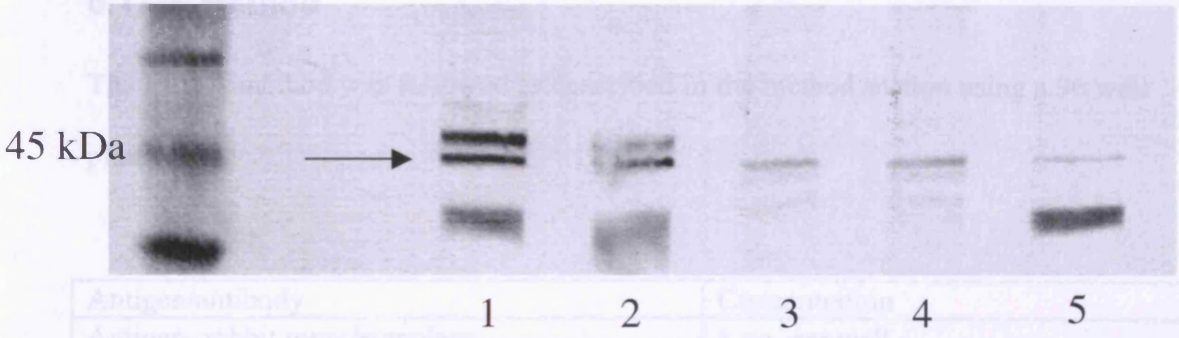


Figure 6.23. ELISA negative patients used in incubation experiments. JN pre-incubation (lane 1), and post-incubation (lane 2). EW pre-incubation (lane 3), and post-incubation (lane 4). Other positive patient with anti-brain enolase antibodies (lane 5). There is no reduction in anti-enolase antibodies post-incubation.

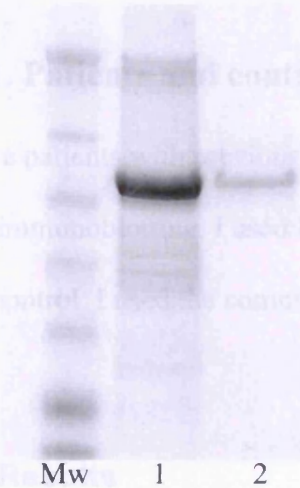


Figure 6.24. Purification of recombinant human alpha enolase. PAGE gel stained with comassie blue, showing recombinant human enolase after His column chelation and elution (lane 1). Lane 2 shows the result of gel digestion. There is reduction in the contaminating E.Coli proteins.

6.17. Development of an ELISA for anti-enolase antibodies using commercial rabbit muscle enolase.

6.17.1. Hypothesis

Rabbit enolase appears to be a potentially useful surrogate of human/rat brain NNE from provisional experiments. Therefore, I aimed to develop an ELISA using rabbit muscle enolase.

6.17.2. Method

The ELISA method was followed as described in the method section using a 96 well plate.

Antigen/antibody	Concentration
Antigen- rabbit muscle enolase	1 µg per well
Primary antibody- patient or control serum	1:200 dilution (200 µl per well)
Secondary antibody- anti-human IgG HRP conjugated	1:1000 (200 µl per well)
Detection- OPD	100 µl per well

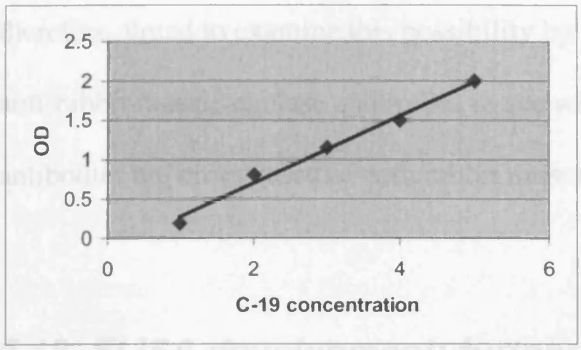
6.17.2.1. Patients and controls

I used five patients with previously described strong anti-NNE antibody binding on Western immunoblotting. I used 8 healthy normal child controls for comparison. As a positive control, I used the commercial anti-enolase C-19 antibody.

6.17.3. Results

6.17.3.1. Commercial antibody (C-19)

Using the commercial antibody at different concentrations, I created a standard curve with the rabbit enolase antigen.

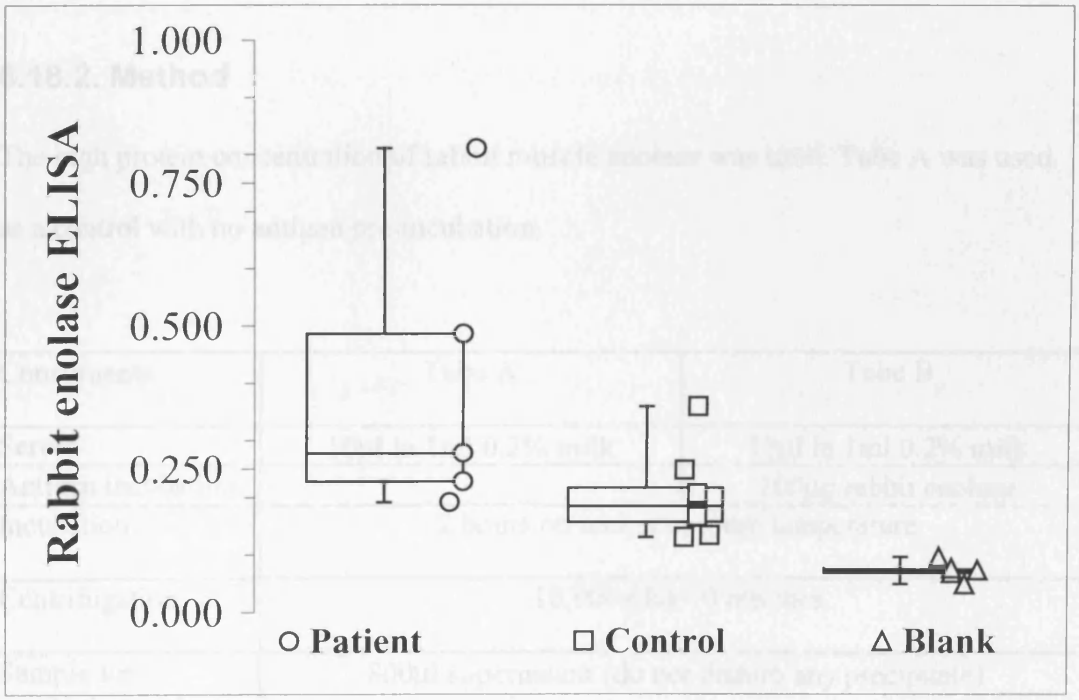


1. blank
2. 1:4000
3. 1:2000
4. 1:1000
5. 1:500

DILUTIONS

Next, I ran the patients and controls and plotted the patients, controls and blanks as follows:

Figure 6.22. ELISA against rabbit muscle enolase, comparing patients against controls and blanks.



As can be seen, although the Patients appear to have elevated anti-enolase antibodies compared to controls, 3 of the patients do not have significantly elevated anti-enolase

antibodies using this assay, although previous experiments have shown that they do have anti-alpha enolase antibodies against human and rat brain. It is possible that not all of the patient's anti-NNE antibodies cross-react with the rabbit muscle enolase. I therefore aimed to examine this possibility by testing the two patients with the lowest anti-rabbit muscle enolase antibodies to see whether these patient's anti-NNE antibodies are cross-reactive with rabbit muscle enolase.

6.18. ELISA development: further absorption experiments.

6.18.1.Hypothesis and aim

To perform absorption experiments using patients previously shown to have anti-NNE antibodies who were negative using the rabbit muscle enolase ELISA. It is suspected that these patient's anti-NNE antibodies do not cross-react with rabbit muscle enolase

6.18.2. Method

The high protein concentration of rabbit muscle enolase was used. Tube A was used as a control with no antigen pre-incubation.

Constituents	Tube A	Tube B
Serum	10µl in 1ml 0.2% milk	10µl in 1ml 0.2% milk
Antigen incubation	-	100µg rabbit enolase
Incubation	2 hours on rocker at room temperature	
Centrifugation	10,000g for 10 minutes	
Sample for incubation with rat enolase	800µl supernatant (do not disturb any precipitate)	

Sera from EW and JN were used (2 ELISA negative patients). The post-incubation sera were incubated with rat brain antigen (ammonium sulphate precipitation) after Western immunoblotting.

6.18.3. Results

There was no alteration in the pre and post incubation anti-brain enolase antibodies of both patients EW and JN (Figure 6.23). This suggests that the anti-NNE antibodies do not cross react with rabbit muscle enolase in these patients.

6.18.4. Conclusions

Although anti-NNE antibodies in some patients cross-react with rabbit muscle enolase, other patients do not. It therefore appears that rabbit muscle enolase will not make a useful surrogate antigen for ELISA or Western blotting assays. It will therefore be necessary to use a brain NNE from rat or human. This will require purification of brain enolases from brain homogenates, or production of a recombinant NNE.

6.19. Purified recombinant human non-neuronal enolase

6.19.1. Introduction

Rabbit muscle enolase, although initially appealing as a measure of anti-NNE antibodies turned out to be a poor surrogate marker. It was therefore necessary to use a human form of NNE. Commercial human NNE purified from human brain is very expensive (£450 for 50 μg) and thus not a practical antigen for using in a diagnostic

assay. We therefore tried to develop a recombinant form of human NNE. Recombinant human NNE was made by Paul Candler (PhD student, Department of Neuroinflammation). He created recombinant human alpha enolase using gene specific primers to amplify mRNA from healthy human lymphocytes, and expressed the protein in an E.Coli vector. The protein was His tagged and partially purified using His chelating columns. Although the human NNE was the dominant protein, there were contaminating E.Coli proteins. I therefore tried to reduce the contaminating proteins with further protein purification techniques. I tried ion exchange chromatography that, although effective, resulted in a poor yield of protein. I therefore simply extracted the NNE protein from PAGE gels as follows:

6.19.2. Method

The His column purifed extract was incubated with SDS and DDT using the standard PAGE sample preparation. The SDS sample was then run in a 10 well PAGE gel, with 2 lanes containing the molecular weight marker. Once the gel had been run for 39 minutes using the standard PAGE method, the gel was removed from the plastic housing. Then a section of gel containing the recombinant human enolase was excised from the gel. I excised the section between 40 and 55kDa. The gel was then minced and incubated in 1 ml of sodium phosphate buffer with 0.1% SDS for 30 minutes at 65^0 C. The gel was then loaded into a microcon concentrator with a membrane cut-off of 100 kDa. The NNE should then flow through the membrane, whereas the gel structure remains above the membrane. The supernatant was then collected and concentrated using Microcon YM-10 concentrators (10 kDa cut-off). The resulting protein was then run in a PAGE gel and compared with the His-purified recombinant NNE.

6.19.3. Results

The gel digestion produced significant improvement in purity, compared to the His column fraction (Figure 6.24).

6.20. Anti-NNE antibody screening of patients and controls using recombinant human NNE.

6.20.1. Method

I now aimed to test a number of patients and controls against recombinant human NNE using Western blotting. The method was as follows:

Patients and controls	20 patients with post-streptococcal CNS disorders (10 SC, 10 PANDAS) 20 controls (10 with neurological disease, 10 healthy children) Positive control: commercial anti-enolase 1:2000
Antigen	60 µg recombinant human NNE per 2D gel
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1:1000 HRP. Colormetric development Anti-Goat IgG 1:1000 for anti-enolase antibody

6.20.2. Results

The patients reacted significantly more compared to the controls (Table 6.a.).

Table 6.a. Anti-recombinant human alpha enolase antibodies in patients compared to controls.

Group	Positive binding (%)
Patients (n=20)	7 of 20 (35%)
Controls (n=20)	0 of 20 (0%)

6.20.3. Conclusion

Patients with post-streptococcal CNS disorders have a higher incidence of antibodies against human NNE than controls. However, a significant number of patients do not have these antibodies. This may be partly attributable to the sensitivity of this assay. The recombinant human protein has an advantage over commercially available enolases due to being identical to human enolase at the genomic and proteomic level. The sensitivity and specificity of anti-neuron-specific enolase antibodies was measured by Andrew Church (as part of his thesis).

6.21. Summary of enolase autoantigens.

Table 6.b. Summary of 45 and 98 kDa proteins

Characteristic	45 kDa upper doublet	45 kDa lower doublet	98 kDa
Mass spectrometry identity	Non-neuronal enolase monomer	Neuron-specific enolase monomer	Neuron-specific enolase dimer
Confirmation with commercial human antigen	+	+	-
Confirmation with recombinant human antigen	+	-	-
Confirmation with commercial antibody	+	+	+

The criticisms and future directions of this work are detailed in the discussion.

Chapter 7. Proteomic identification of the 40 kDa autoantigen

7.1. Choosing the antigen: Rat brain and human basal ganglia.

7.1.1. Aim

The aim of this section was to purify and identify the 40 kDa autoantigen. Firstly, it was necessary to make sure that I could use rat brain tissue instead of human brain tissue as the brain homogenate.

7.1.2. Method

1. The rat brain supernatant homogenate was used as previously described.
2. Western blotting was performed using 4-12% Bis-Tris PAGE gel. 'See Blue plus 2' molecular weight marker was loaded in lane 1. 3 µg of Human basal ganglia was loaded in lane 2. 15 µG of rat brain was loaded in lane 3.
3. The gel was electrophoresed and transferred using the standard method.
4. A patient with strong 40 kDa antibodies (MB) was incubated overnight and washed as per standard method.
5. The nitrocellulose was developed colormetrically.

7.1.3. Results

The 40 kDa autoantigen was present in both rat brain and was the same molecular weight as the 40 kDa autoantigen in human basal ganglia (Figure 7.1).

7.2. Regional localisation of 40kDa antigen in rat tissues

7.2.1. Aim

In order to learn more about the 40 kDa antigen, I aimed to determine the regional localisation of the protein in rat tissues. Rat brain (with cerebellum removed), cerebellum, kidney, liver and heart were used.

7.2.2. Method

1. One 10-lane 4-12% Bis-Tris gel was used.
2. The rat tissues were homogenised in the same way, and total proteins were measured. The proteins were then normalised, and 75µg of each homogenate was loaded into a separate lane.
3. The gel was electrophoresed using the normal method, transferred to nitrocellulose and blocked with 2% milk for 2 hours.
4. A patient with strong 40 kDa antibodies was incubated 1:300 overnight.
5. The blot was then washed with 10 changes of 0.025% Tween/0.2% milk in saline.
6. A secondary antibody (rabbit anti-human IgG with conjugated HRP 1:1000) was incubated for 2 hours, and then the blot was washed as before.

7. The blot was then developed colormetrically for 15 minutes.

7.2.3. Results

The 40 kDa protein is present in rat brain and heart. The protein may also be weakly present in kidney and liver (Figure 7.2.).

7.3. Ammonium sulphate fractionation

7.3.1. Aim

I aimed to use protein purification methods to purify the 40 kDa autoantigen in order to identify the protein using mass spectrometry. In the first instance, I used ammonium sulphate fractionation as a first step in protein purification.

7.3.2. Method

1. The ammonium sulphate fractionation method as previously described was followed. In summary, rat brain was homogenised as previously described.
2. The following fractions were precipitated, then re-suspended in 1ml of sodium phosphate buffer Ph 7.0: 0-20%

   20-40%

   40-60%

   60-80%

   80-100%

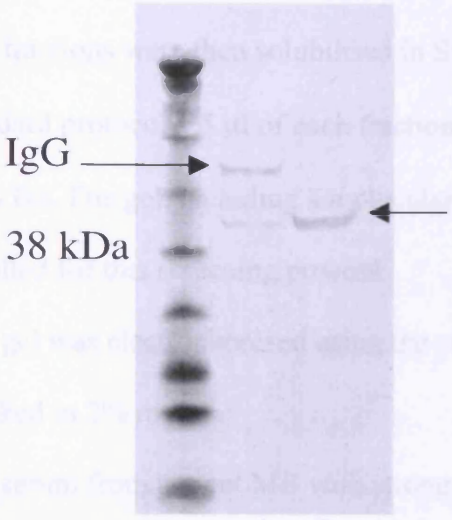


Figure 7.1. Comparison of human basal ganglia and rat brain (40 kDa antigen). The 40 kDa antigen was present in both rat brain (lane 3) and human basal ganglia (lane 2). Human IgG was present in the human basal ganglia preparation only.

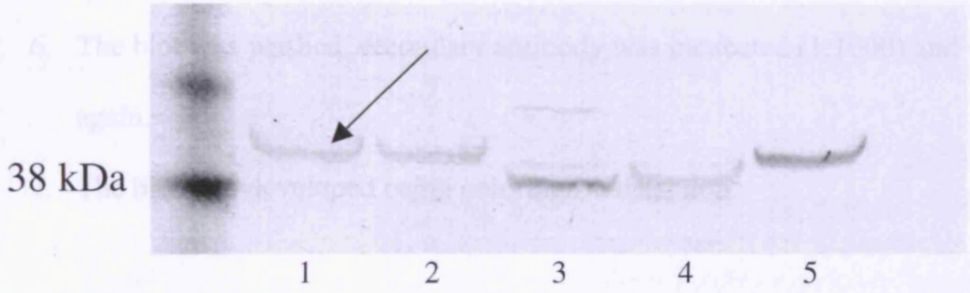


Figure 7.2. Regional comparison of 40 kDa antigen. Lane 1: Rat brain (minus cerebellum). Lane 2: Rat cerebellum. Lane 3: Rat kidney. Lane 4: Rat liver. Lane 5: Rat heart. The 40 kDa antigen was present and enriched in rat brain and heart. The protein was possibly weakly present in liver and kidney. There was a strong band (38kDa) in both kidney and liver, which may be irrelevant, or represent an isoform of the brain protein.

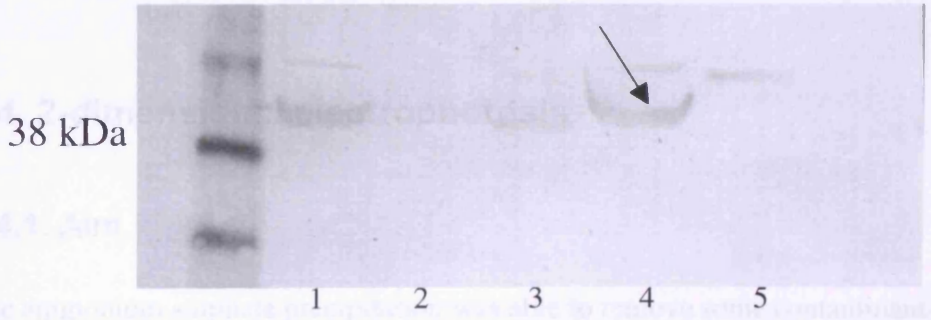


Figure 7.3. Ammonium sulphate fractionation. Lane 1: Whole rat brain. Lane 2: 0-20% fraction. Lane 3: 20-40% fraction. Lane 4: 40-60% fraction. Lane 5: 60-80% fraction. Lane 6: 80-100% fraction. The 40 kDa protein was most abundant in the 40-60% fraction. This patient also had anti-45 kDa antibodies (possibly enolase).

3. The fractions were then solubilised in SDS and reduced with DDT using the standard protocol. 25 µl of each fraction was then loaded onto a 10-lane 4-12% Bis-Tris gel, including a molecular weight marker. The samples were not desalted for this screening process.
4. The gel was electrophoresed using the standard protocol, transferred, and blocked in 2% milk.
5. The serum from patient MB with strong anti-40 kDa antibodies was incubated at 1:300 concentration overnight.
6. The blot was washed, secondary antibody was incubated (1:1000) and washed again.
7. The blot was developed using colormetric detection.

7.3.3. Results

The 40 kDa antigen was most prevalent in the 40-60% fraction (Figure 7.3). This fraction was used for further protein purification strategies.

7.4. 2-dimensional electrophoresis

7.4.1. Aim

The ammonium sulphate precipitation was able to remove some contaminants and concentrate the antigen of interest in the precipitant. However, this method did not achieve adequate purification alone. 2-dimensional electrophoresis enabled further protein separation by weight and charge.

7.4.2. Method

1. The 2-dimensional method was employed as previously described.
2. First, the 40-60% ammonium sulphate fraction was desalted (method) into sodium phosphate buffer, pH 7.0. This buffer allows proteins to exist in their isoelectric form. The 40-60% fraction was then concentrated using micropore YM-10 devices. The sample was then made up in IEF buffer and a small amount of SDS to achieve a final SDS concentration of 0.05% SDS (112.5µl sample, 12.5µl 1%SDS, 125µl IEF buffer).
3. The method was then followed exactly as previously specified.
4. After the second stage, one gel was silver stained as described. A further gel was transferred to nitrocellulose and blotted in the normal way.
5. The blot was incubated with patient serum (MB) at 1:300 dilution overnight. The blot was then washed, secondary antibody incubated and washed again as per usual Western blotting protocol.

7.4.3. Results

The silver stained gel and Western blot results are presented in figure 7.4. As can be seen, it was possible to identify a candidate protein from 2-dimensional electrophoresis. This protein was subjected to gel digest and Q-TOF mass spectrometry.

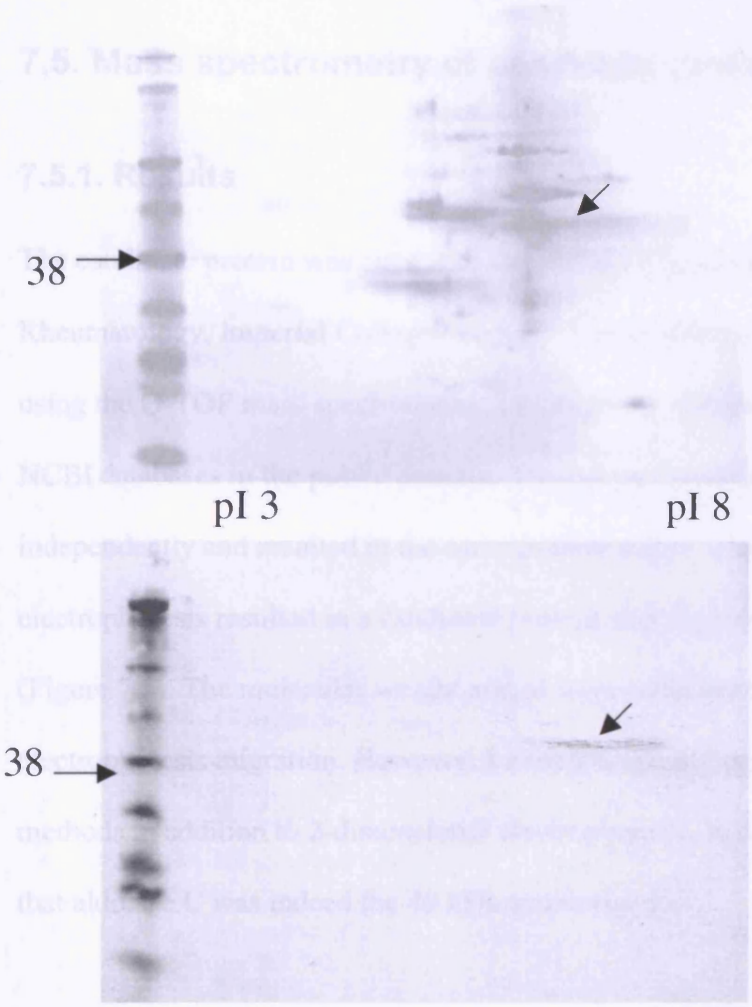


Figure 7.4. 2-dimensional electrophoresis showing silver stained gel (above), and Western blot (below) after incubation with MB serum. The candidate antigen is labelled with the arrow.

Protein	Score	Coverage	Molecular weight	Pi
Fructose-bisphosphate aldolase C (Aldolase C)	2066	40.0%	39134 Da	7.1

ALFC_RAT
Fructose-bisphosphate aldolase C (EC 4.1.2.13) (Br

(P09117)

```
  1     PHSYPALSAE QKKELSDIAL RIVAPGKGIL AADESVGSMA KRLSQIGVEN TEENRRLYRQ VLFSADDRVK
 71     KCIGGVIFFH ETLYQKDDNG VPFVRTIQEK GILVGIKVDK GVVPLAGTDG ETTTQGLDGL LERCAQYKKD
141     GADFAKWRCV LKISDRTPSA LAILENANVL ARYASICQQN GIVPIVEPEI LPDGDHDLKR CQFVTEKVLA
211     AVYKALSDHH VYLEGTLLKP NMVTPGHACP IKYSPEEIAM ATVTALRRTV PPAVPGVTFL SGGQSEEEAS
281     LNLNAINRCS LPRPWALTFS YGRALQASAL SAWRGQRDNA GAATEEFIKR AEMNGLAAQG KYEGSGDGGA
351     AAQSLYVANH AY
```

Figure 7.5. Mass spectrometry result of 40 kDa protein.

7.5. Mass spectrometry of candidate protein using Q-TOF.

7.5.1. Results

The candidate protein was cut out of the gel and digested by Robin Wait (Institute of Rheumatology, Imperial College London). The resulting peptides were then processed using the Q-TOF mass spectrometer. The resulting spectrum was compared with NCBI databases in the public domain. The tail and head of the protein were processed independently and resulted in the same protein match. The 2-dimensional electrophoresis resulted in a candidate protein with high mass spectrometry coverage (Figure 7.5). The molecular weight and pI were consistent with the 2-dimensional electrophoresis migration. However, I aimed to use different protein purification methods in addition to 2-dimensional electrophoresis, in order improve confidence that aldolase C was indeed the 40 kDa autoantigen.

7.6. Identifying the 40 kDa autoantigen using chromatography

7.6.1. Aim

2-dimensional electrophoresis and mass spectrometry identified aldolase C as the candidate 40 kDa autoantigen. Before significant amounts of time and money in creating recombinant aldolase C, I wanted to confirm these findings using different purification techniques, namely chromatography. I found that performing ammonium sulphate precipitation followed by hydrophobic interaction chromatography then ion exchange chromatography produced good separation for identification.

7.6.2. Method

1. HIC 5ml butryl sepharose FF high performance column was used. Phenyl FF columns were less successful in screening.
2. The sample (40-60% ammonium sulphate fraction) was first desalted into the start buffer (sodium phosphate pH 7.0 buffer with added 1.5 Molar ammonium sulphate). The elution buffer was sodium phosphate pH 7.0 alone.
3. The FPLC was programmed using the'HIC template' and followed as previously described.

Variable	Value
Flow rate	0.25 ml/min
Wash out after sample injection	5 column volumes
Length of gradient	20 column volumes
Fraction size	1ml

4. I then Western blotted different fractions on a 4-12% Bis-Tris 10-lane gel. The proteins were transferred to nitrocellulose and Western blotted using the standard method.
5. Serum from patient MB (anti-40 kDa antibodies) was used as the primary antibody. Anti-human IgG HRP conjugated 1:1000 was used as the secondary antibody.
6. The blot was developed colormetrically.

7.6.3. Result

The hydrophobic interaction chromatography produced reasonable separation of proteins. This method was performed rapidly and over a short gradient. The aim was then to proceed to a further purification step, namely ion exchange chromatography. Western blotting showed that the 40 kDa protein was present in lane 4 during HIC (Figure 7.6.). This fraction was therefore used in further purification chromatography.

7.7. Further purification of the 40 kDa protein using ion exchange chromatography.

7.7.1. Aim

The hydrophobic interaction chromatography provided a useful first stage in the purification strategy. I next used this fraction for ion exchange chromatography.

7.7.2. Method

1. I used the 5ml HiTrap Q fast flow column and connected the column to the FPLC using the standard technique. The binding buffer was Tris-HCl buffer (pH 8.0), and the elution buffer was Tris-HCl buffer (pH 8.0) with 1.5 Molar NaCl.
2. I first desalted the lane 4 fraction from the HIC method into the IEX binding buffer.
3. I then loaded the sample onto the FPLC as previously described. I used the following variables:

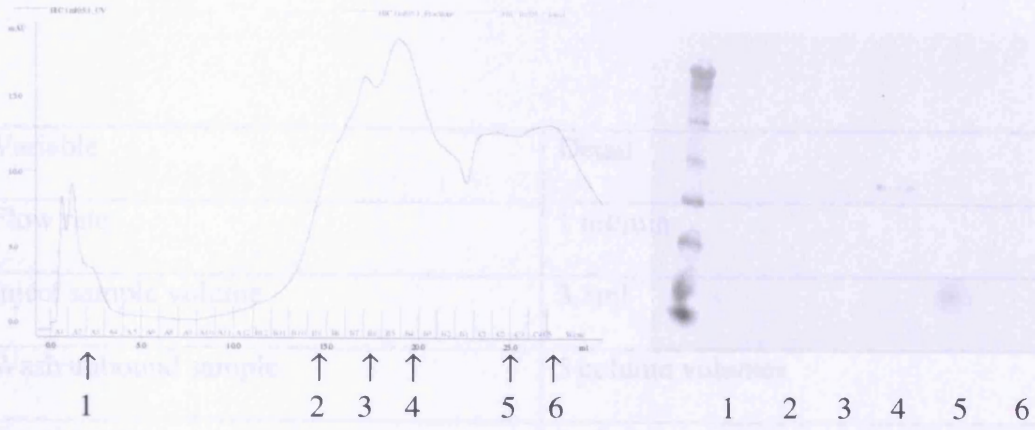


Figure 7.6. Hydrophobic interaction chromatography (40 kDa antigen). The 40 kDa protein was present in lane 4 during Western blotting which equated to fraction B5 during hydrophobic interaction chromatography.

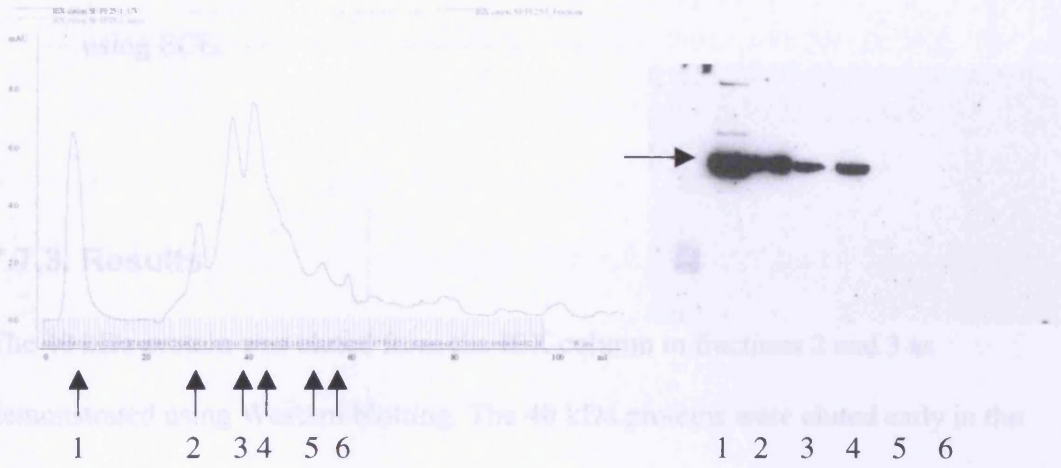


Figure 7.7. The ion exchange chromatography produced further separation of the 40 kDa proteins. The protein was eluted from the column early in the gradient (fractions 2 and 3). Some of the protein did not bind and flowed through the column (fraction 1).

Variable	Detail
Flow rate	1 ml/min
Inject sample volume	3.3ml
Wash unbound sample	5 column volumes
Fraction size	1ml
Gradient	0-100% in 15 column volumes

4. The chromatogram shown in Figure 7.7 was produced, and fractions were selected for testing as indicated (arrows). The samples were loaded onto a 10-well gel and submitted to PAGE electrophoresis as previously described. The gel was Western blotted, incubated with patient MB (1:300), and developed using ECL.

7.7.3. Results

The 40 kDa protein was eluted from the IEX column in fractions 2 and 3 as demonstrated using Western blotting. The 40 kDa proteins were eluted early in the IEX protocol (chromatogram and Figure 7.7)

7.7.4. Conclusion

The 40 kDa protein had been partially purified using ammonium sulphate precipitation, hydrophobic interaction chromatography and ion exchange chromatography. I therefore performed a silver stain of the fraction in an attempt to identify the protein using mass spectrometry.

7.8. Identifying the 40 kDa protein after chromatography purification using mass spectrometry.

7.8.1. Aim

Next, I silver stained the final fraction in an attempt to identify the 40 kDa protein using mass spectrometry.

7.8.2. Method

1. The following fractions were run on a 10-well 4-12% Bis-Tris gel using PAGE. One gel was Western blotted and incubated with MB (1:300). The other gel was silver stained for identification of the 40 kDa protein.
    - Ammonium sulphate fraction (40-60%)
    - Hydrophobic interaction chromatography fraction (lane 4 fraction from HIC)
    - Ion exchange chromatography (fraction 3 from IEX)

7.8.3. Results

The Western blot confirmed that the 40 kDa protein was maintained in the purification procedure (Figure 7.8). The silver stain showed that the 40 kDa protein is partially purified by these methods, with reduction in contaminating proteins. The 40 kDa protein was submitted to mass spectrometry.

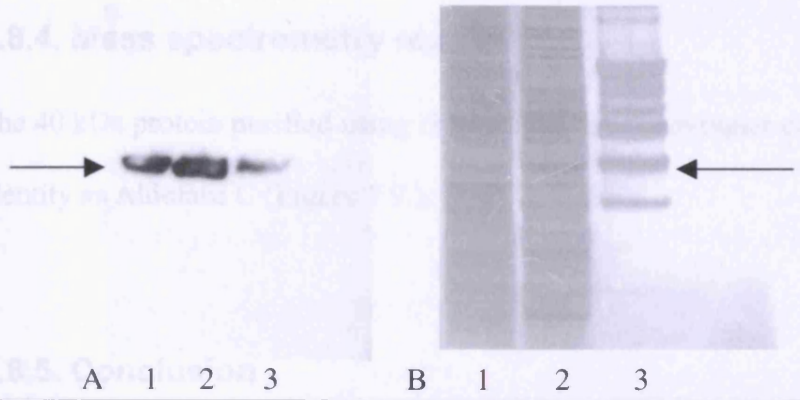


Figure 7.8. 40 kDa antigen purification after chromatography.Western blot (A) and Silver stain (B) of ammonium sulphate precipitation 40-60% fraction (lane 1), hydrophobic interaction chromatography fraction (lane 2), and ion exchange chromatography (lane 3). The 40 kDa protein is partially purified by this procedure with reduction in contaminating proteins.

Protein	Score	Coverage	Molecular weight	Pi
Fructose-bisphosphate aldolase C (Aldolase C)	1066	16.8%	39134 Da	7.1

ALFC_RAT
Fructose-bisphosphate aldolase C (EC 4.1.2.13) (Br

(P09117)

```
1       PHSYPALSAE QKKELSDIAL RIVAPGKGIL AADESVGSMA KRLSQIGVEN TEENRRLYRQ VLFSADDRVK
71      KCIGGVIFFH ETLYQKDDNG VPFVRTIQEK GILVGIKVDK GVVPLAGTDG ETTTQGLDGL LERCAQYKKD
141     GADFAKWRCV LKISDRTPSA LAILENANVL ARYASICQQN GIVPIVEPEI LPDGDHDLKR CQFVTEKVLA
211     AVYKALSDHH VYLEGTLLKP NMVTPGHACP IKYSPEEIAM ATVTALRRTV PPAVPGVTFL SGGQSEEEAS
281     LNLNAINRCS LPRPWALTFS YGRALQASAL SAWRGQRDNA GAATEEFIKR AEMNGLAAQG KYEGSGDGGA
351     AAQSLYVANH AY
```

Figure 7.9. Mass spectrometry result of 40 kDa protein.

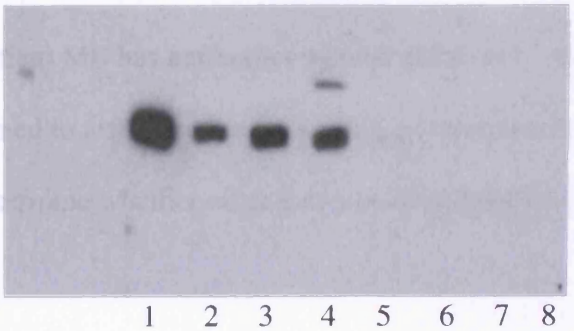


Figure 7.10. Other patients with anti-aldolase antibodies. Lane 1: anti-aldolase commercial antibody 1:5000. Lane 2-5: Patients. Lanes 6-8: Controls. The patients all bind to aldolase C and the reactivity lines up with the commercial antibody. The controls show no reactivity.

7.8.4. Mass spectrometry results

The 40 kDa protein purified using chromatographic techniques confirmed the protein identity as Aldolase C (Figure 7.9.).

7.8.5. Conclusion

Aldolase C was confirmed as the likely autoantigen in this patient. Aldolase C was purified in 2 independent methods (2-dimensional electrophoresis and chromatography). Aldolase C is the neuronal isoform of aldolase. Like enolase, aldolase exists in the cytoplasm of neurones. Like enolase, aldolase also exists on the neuronal membrane surface (Knull HR and Fillmore SJ, 1985) and is suspected to be involved in redox control of receptor function and signal transduction (Bulliard C et al., 1997).

7.9. Testing the findings using commercial anti-aldolase antibodies

7.9.1. Aim

Patient MB has antibodies against aldolase C, the brain isoform of aldolase. I next aimed to test these findings using a commercial anti-aldolase antibody, and also determine whether other patients have these antibodies.

7.9.2. Method

1. Using Western immunoblotting, I used the partially purified aldolase C fraction. This fraction had been partially purified by ammonium sulphate fractionation, hydrophobic interaction chromatography and ion exchange chromatography. This fraction was concentrated using the YM-10 micropore. The sample was then loaded onto a 2D 4-12% Bis-Tris gel and electrophoresed, transferred to nitrocellulose and blocked using the standard method.
2. As a positive control, I used commercial anti-human aldolase antibodies that have been previously shown to bind to human brain aldolase C (AbCam, UK).
3. I then ran 4 patients that I have previously identified with probable 40 kDa autoantibodies (1:300 dilution). I compared the findings with 3 healthy controls.
4. The blot was washed, secondary antibody applied (anti-human 1:5000), and washed again using the standard method.
5. The blot was developed using ECL.

7.9.3. Result

The 4 patients all have anti-aldolase C antibodies (lane 5 weakly) (Figure 7.10). The 3 controls are all negative. This encouraging result needs to be furthered with other patients and controls. The commercial anti-aldolase antibody lines up with the patients, further confirming the identity of the 40 kDa protein as Aldolase C.

7.10. Testing the patients with anti-aldolase C antibodies against recombinant human Aldolase C.

7.10.1. Method

I now aimed to test a number of patients and controls against human aldolase C using Western blotting. Recombinant human Aldolase C was made by Paul Candler at the Institute of Neurology. In brief, the protein is made by E.Coli after inserting the human aldolase C genome into the E.Coli vector. The protein was His-Tagged, and purified using a His column. Despite good purification, there was a low level of contaminating E.Coli proteins. I used a commercial anti-aldolase antibody as a positive control. The method was as follows:

Patients and controls	20 patients with post-streptococcal CNS disorders (10 SC, 10 PANDAS) 20 controls (20 healthy children) positive control: commercial anti-aldolase 1:5000
Antigen	100 µg recombinant human aldolase C per 2D gel
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1:1000 HRP. Colormetric development Anti-Goat IgG 1:1000 for anti-aldolase antibody

7.10.2. Results

The patients reacted significantly more compared to the controls (Table 7.a.). One control bound to human aldolase C.

Table 7.a. Anti-human aldolase C antibodies in patients compared to controls.

Group	Positive binding (%)
Patients (n=20)	7 of 20 (35%)
Controls (n=20)	1 of 20 (5%)

7.10.3. Conclusion

More patients reacted with the human aldolase C than the controls, however, most of the patients did not react with this protein. This may be at least partly related to the sensitivity setting of this methodology. It is possible that the assay is too insensitive. This will be discussed in more detail in the discussion.

Chapter 8. Proteomic identification of the 60 kDa protein

8.1. Choosing the antigen: rat brain and human basal ganglia

8.1.1. Aim

The aim of this section was to purify and identify the 60 kDa autoantigen. Firstly, it was necessary to make sure that I could use rat brain tissue rather than human brain tissue as the brain homogenate.

8.1.2. Method

1. The rat brain supernatant homogenate was used as previously described.
2. Western blotting was performed using 4-12% Bis-Tris PAGE gel. 'See Blue plus 2' molecular weight marker was loaded in lane 1. 3 μg of Human basal ganglia was loaded in lane 2. 15 μG of rat brain was loaded in lane 3.
3. The gel was electrophoresed and transferred using the standard method.
4. A patient with strong 60 kDa antibodies was incubated overnight and washed as per standard method.
5. The nitrocellulose was developed colormetrically.

8.1.3. Results

The 60 kDa autoantigen is present in both rat brain and is the same molecular weight as the 60 kDa autoantigen in human basal ganglia (figure 8.1).

8.2. Regional localisation of 60kDa antigen in rat tissues

8.2.1. Aim

In order to learn more about the 60 kDa antigen, I aimed to determine the regional localisation of the protein in rat tissues. Rat brain (with cerebellum removed), cerebellum, kidney, liver and heart were used.

8.2.2. Method

1. One 10-lane 4-12% Bis-Tris gel was used.
2. The rat tissues were homogenised in the same way, and total proteins were measured. The proteins were then normalised, and 25μg of each homogenate was loaded into a separate lane. A molecular weight marker was loaded in lane 1.
3. The gel was electrophoresed using the normal method, transferred to nitrocellulose and blocked with 2% milk for 2 hours.
4. A patient with strong 60 kDa antibodies was incubated 1:300 overnight.
5. The blot was then washed with 10 changes of 0.025% Tween/0.2% milk in saline.
6. A secondary antibody 1:1000 (rabbit anti-human IgG with HRP conjugation) was incubated for 2 hours, and then the blot was washed as before.
7. The blot was then developed colormetrically for 15 minutes.

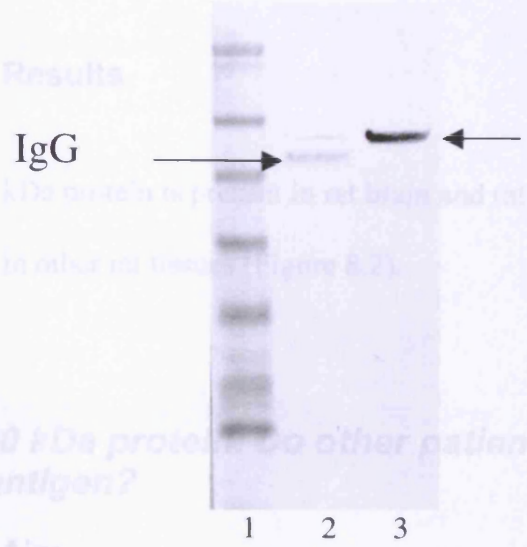


Figure 8.1. Human basal ganglia and rat brain comparison. The 60 kDa antigen is present in both rat brain and human basal ganglia (arrowed). Human IgG is present in the human basal ganglia preparation only (IgG arrowed). The proteins have the same molecular weight.

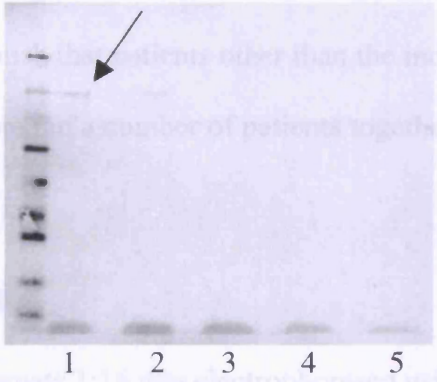


Figure 8.2. Regional localisation of the 60 kDa protein. Lane 1: Rat brain (minus cerebellum). Lane 2: Rat cerebellum. Lane 3: Rat kidney. Lane 4: Rat liver. Lane 5: Rat heart. The 60 kDa antigen is present and enriched in rat brain and weakly in heart.

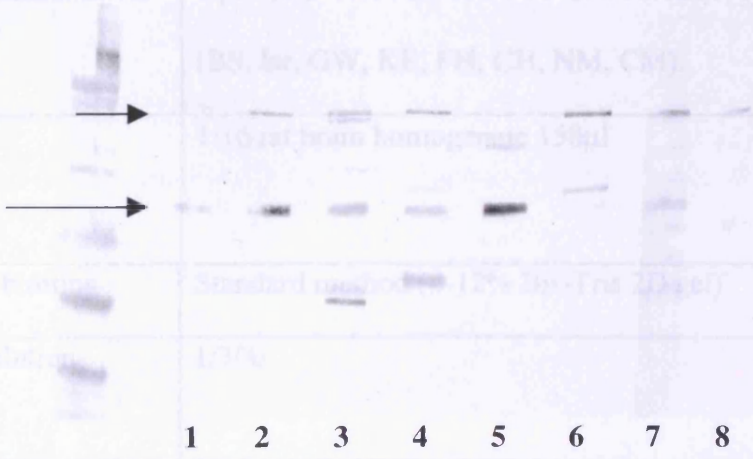


Figure 8.3. Different patients with antibodies to 60 kDa protein. Patients in lane 1-5 and 7 appear to have the same 60 kDa autoantigen (long arrow). 98 kDa autoantigen antibodies are also common in these patients (short arrow).

8.2.3. Results

The 60 kDa protein is present in rat brain and rat cerebellum, very weakly in heart, but not in other rat tissues (Figure 8.2).

8.3. 60 kDa protein. Do other patients react with the same autoantigen?

8.3.1. Aim

I wanted to establish that patients other than the index case reacted with the same protein. I therefore ran a number of patients together on a Western blot against whole rat brain.

8.3.2. Method

Rat brain homogenate 1:16 was electrophoresed using the standard method and Western blotting as previously described.

Patients and controls	8 patients with suspected 60 kDa autoantibodies (BS, Isr, GW, KE, FH, CH, NM, CM).
Antigen	1:16 rat brain homogenate 150µl
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1/1000. Colormetric development

8.3.3. Results

Different patients reacted with the same 60 kDa autoantigen (lanes 1-8, particularly 1-5 and 7 in Figure 8.3).

8.4. Identifying the 60 kDa protein

8.4.1. Aim

To use protein purification methods to purify the 60 kDa autoantigen in order to identify the protein using mass spectrometry.

8.4.2. Ammonium sulphate fractionation

8.4.2.1. Method

The ammonium sulphate fractionation method as previously described was followed.

8.4.2.2. Results

Fortuitously, the 60kDa protein was enriched in the 40-60% fraction, as was true of the 40 and 45 kDa proteins. The 40-60% fraction was therefore used for further purification strategies.

8.4.3. Chromatography

8.4.3.1. Aim

I next used the 40-60% fraction for further purification strategies. I suffered a number of false starts with further separation. Ion exchange chromatography lead to co-precipitation with another protein which caused initial confusion. However, using hydrophobic interaction chromatography first proved to be a better and significantly purifying step rather than ion exchange chromatography.

8.4.3.2. Method

1. HIC 5ml butryl sepharose FF high performance column was used.
2. The sample (40-60% ammonium sulphate fraction) was first desalted into the start buffer (sodium phosphate pH 7.0 buffer with added 1.5 Molar ammonium sulphate). The elution buffer was sodium phosphate pH 7.0 alone.
3. The FPLC was programmed using the 'HIC template'.

Variable	Value
Flow rate	1 ml/min
Wash out after sample injection	5 column volumes
Length of gradient	30 column volumes
Fraction size	2ml

4. I then Western blotted different fractions on a 4-12% Bis-Tris 10-lane gel (Figure 8.4.). The proteins were transferred to nitrocellulose and Western blotted using the standard method.

5. Serum from patient Isr (anti-60 kDa antibodies) was used as the primary antibody. Anti-human IgG HRP conjugated 1:5000 was used as the secondary antibody.
6. The blot was developed with ECL.

8.4.3.3. Result

The hydrophobic interaction chromatography produced surprisingly good separation of proteins. This method was performed slowly aiming to achieve good separation. After Western blotting, it showed that the 60 kDa protein was present in fraction E10 (lane 2)

in Figure 8.4.

8.5. Identification of the candidate protein using mass spectrometry

8.5.1. Method

The fraction E10 (lane 2) was submitted to PAGE with a control lane (rat brain fraction).

8.5.2. Results

The HIC fraction E10 was loaded onto lane 2 of the PAGE gel (Figure 8.5). The 60 kDa protein was enriched in this fraction with significant reduction in contaminating proteins. The protein was cut out by Robin Wait, digested and subjected to Q-TOF mass spectrometry and identified as pyruvate kinase M1 (Figure 8.6).

Pyruvate kinase is an enzyme involved in energy metabolism that resides both in the cytoplasm and on the neuronal surface, and has a molecular mimic in streptococci.

The M1 isoezyme is expressed in brain and heart.

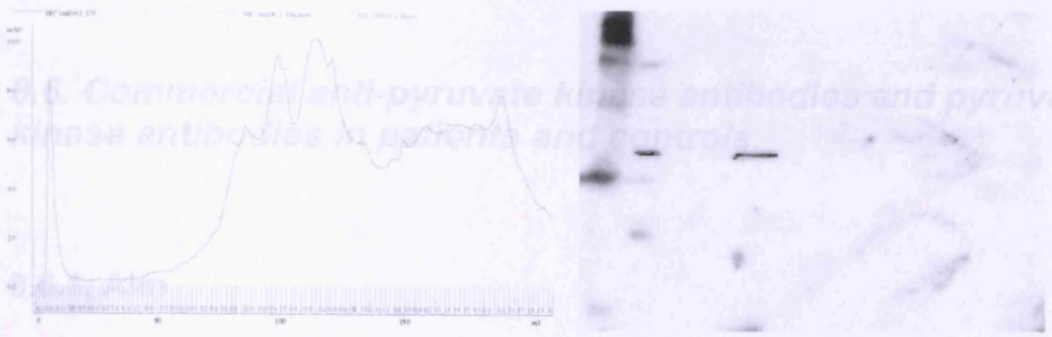


Figure 8.4. HIC chromatography of 60 kDa protein. HIC produced good separation of proteins. The 60 kDa protein was eluted early (fraction 2).

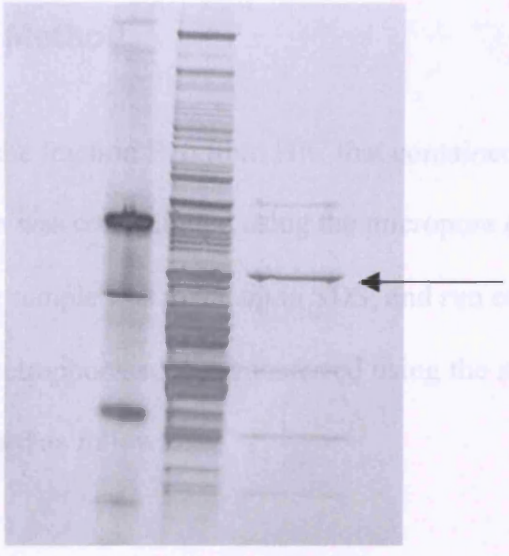


Figure 8.5. Purification of 60 kDa protein. Rat brain 40-60% fraction (lane 1) and HIC fraction 'E10' (lane 2) were separated by PAGE. The protein was labelled with a long arrow.

Protein	Score	Coverage	Molecular weight	Pi
Pyruvate kinase, M1 isoform	2415	20.5%	57668 Da	7.0

KPY1_RAT
Pyruvate kinase, M1 isozyme (EC 2.7.1.40) (Pyruvat

(P11980)

```
  1    PKPDSEAGTA FIQTQQLHAA MADTFLEHMC RLDIDSAPIT ARNTGIICTI GPASRSVEML KEMIKSGMNV
 71    ARLNFSHGTH EYHAETIKNV RAATESFASD PILYRPVAVA LDTKGPEIRT GLIKGSGTAE VELKKGATLK
141    ITLDNAYMEK CDENILWLDY KNICKVVEVG SKIYVDDGLI SLQVKEKGAD YLVTEVENGG SLGSKKGVNL
211    PGAAVDLPAV SEKDIQDLKF GVEQDVDMVF ASFIRKAADV HEVRKVLGEK GKNIKIISKI ENHEGVRRFD
281    EILEASDGIM VARGDLGIEI PAEKVFLAQK MMIGRCNRAG KPVICATQML ESMIKKPRPT RAEGSDVANA
351    VLDGADCIML SGETAKGDYP LEAVRMQHLI AREAEAAVFH RLLFEELARA SSQSTDPLEA MAMGSVEASY
421    KCLAAALIVL TESGRSAHQV ARYRPRAPII AVTRNPQTAR QAHLYRGIFP VLCKDAVLDA WAEDVDLRVN
491    LAMNVGKARG FFKKGDVVIV LTGWRPGSGF TNTMRVVPVP
```

Figure 8.6. Mass spectrometry of the 60 kDa protein.

8.6. Commercial anti-pyruvate kinase antibodies and pyruvate kinase antibodies in patients and controls.

8.6.1. Aim

I next aimed to test the patients with suspected antibodies against rat pyruvate kinase using a commercial anti-pyruvate kinase antibody as a positive control.

8.6.2. Method

I used the fraction E10 from HIC that contained the rat pyruvate kinase. The E10 fraction was concentrated using the micropore concentrators YM-10 from 1ml to 150 µl. The sample was made up in SDS, and run on a 4-12% 2D Bis-Tris gel. The gel was electrophoresed and transferred using the standard method. The blot was then processed as follows:

Patients and controls	4 patients with suspected 60 kDa autoantibodies (Isr, GW, KE, TT) Negative control: secondary antibody only Positive control: Goat anti-pyruvate kinase antibody (1:2000)
Antigen	E10 fraction from HIC 150µl
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300

Antibody detection	Anti-human IgG 1/5000 HRP. ECL development

8.6.3. Results

As can be seen in Figure 8.7, the commercial antibody and patients reacted with the pyruvate kinase in the E10 fraction. The patient with the strongest reactivity on screening (KE) reacted strongest on this Western blot (lane 2). The Western blot was technically slightly untidy, probably related to the small amount of antigen loaded onto the Western blot, with resulting relatively high background reactivity.

8.6.4. Conclusion

I next aimed to test the patients against commercially available pyruvate kinase. The only available pyruvate kinase commercially available was rabbit pyruvate kinase.

8.7. Testing homology of human with commercial pyruvate kinase antigens.

8.7.1. Aim

I bought rabbit pyruvate kinase hoping that this antigen would make a useful surrogate antigen for antigen development. I first compared the homology of rabbit and rat pyruvate kinase with human M1 pyruvate kinase (PYK).

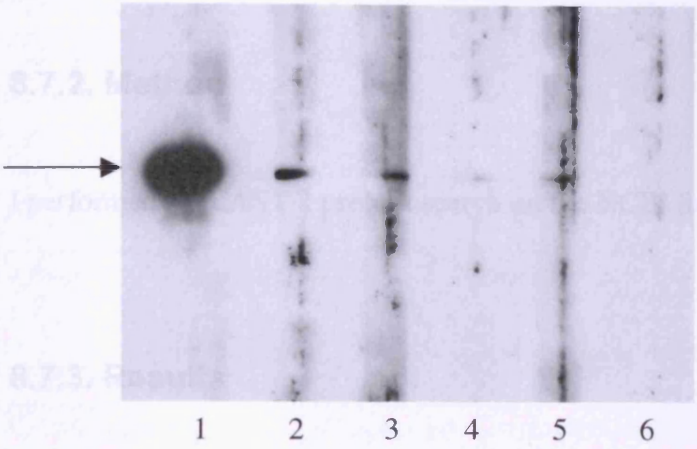


Figure 8.7. The commercial anti-pyruvate kinase antibody (lane 1) strongly reacted with the pyruvate kinase- the antibody was overloaded. Patients (lanes 2-5) also reacted with the pyruvate kinase. Lane 6 was anti-human secondary antibody only showing no reactivity.

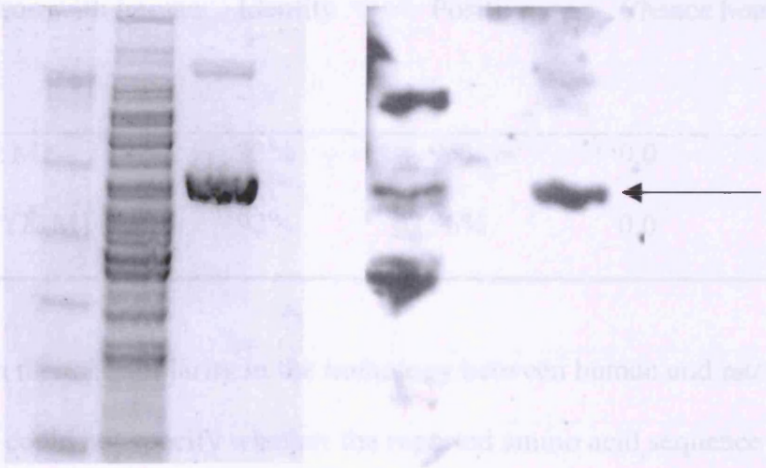


Figure 8.8. Testing the findings with commercial pyruvate kinase antigen. Coumassie stained gel (lanes 1 and 2 with molecular weight marker) show a strong band at 60 kDa in lane 2, and a weaker band at ~100 kDa(presumably the dimer of pyruvate kinase). The Western blot shows reactivity against a 60 kDa protein in the rat brain fraction (lane 3) and to the rabbit PYK (lane 4- arrowed).

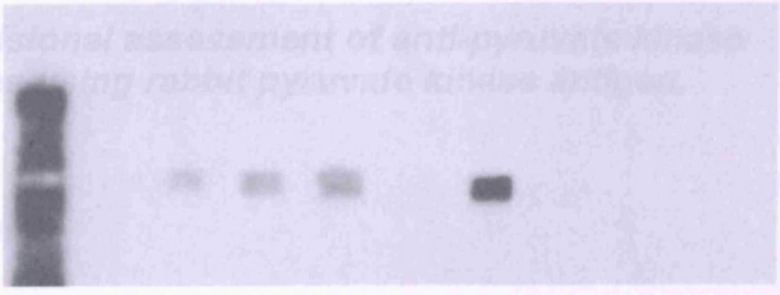


Figure 8.9. Different patients with anti-pyruvate kinase antibodies. Patients (lane 2-5) and controls (lanes 6-8) against rabbit PYK. Commercial antibody in lane 1. Three of four patients are positive, whereas all 3 controls are negative.

8.7.2. Method

I performed a BLAST 2 protein search on the NCBI database.

8.7.3. Results

Comparison with human PYK M1	Identity	Positive	Chance homology
Rat PYK M1	93%	95%	0.0
Rabbit PYK M1	92%	96%	0.0

Although there is similarity in the homology between human and rat/ rabbit tissue, the database could not specify whether the reported amino acid sequence was the brain isoform. The commercial rabbit PYK is derived from muscle, whereas all work so far has used rat brain. The amino acid sequence, conformation and immunogenicity may differ in the muscle and brain.

8.8. Provisional assessment of anti-pyruvate kinase antibodies using rabbit pyruvate kinase antigen.

8.8.1. Method

I first compared rat 40-60% fraction with the commercial rabbit pyruvate kinase (PYK). I performed the experiment as follows:

Patients and controls	3 patients with suspected 60 kDa autoantibodies (Isr, KE, GW)
Antigen	Lane 1: Rat brain 40-60% fraction (25 µg) Lane 2: Rabbit muscle pyruvate kinase (2.5 µg)
Commassie stain	Lanes 1 and 2 were commassie stained using the standard method.
Western blotting	Lanes 3 and 4 were Western blotted using the standard method (4-12% Bis-Tris 10 well gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1/5000 HRP. ECL development

8.8.2. Results

The coummassie stain demonstrated a 60 kDa antigen in lane 2. Western blotting showed reactivity of all 3 patients to a 60 kDa antigen in the rat 40-60% fraction, and also to the rabbit pyruvate kinase (Figure 8.8).

8.8.3. Conclusion

With this encouraging initial result, I aimed to test further patients against the rabbit PYK.

8.9. Anti-pyruvate kinase antibody screening of patients and controls using rabbit pyruvate kinase.

8.9.1. Method

I now aimed to test a number of patients and controls against rabbit pyruvate kinase using Western blotting. The method was as follows:

Patients and controls	20 patients with post-streptococcal CNS disorders (10 SC, 10 PANDAS) 20 controls (10 with neurological disease, 10 healthy children) Positive control: commercial anti-PYK 1:5000
Antigen	20 µg rabbit PYK per 2D gel
Western blotting	Standard method (4-12% Bis-Tris 2D gel)
Serum dilution	1/300
Antibody detection	Anti-human IgG 1:5000 HRP. ECL development Anti-Goat IgG 1:5000 for PYK antibody

8.9.2. Results

The patients reacted significantly more compared to the controls (Table 8.a.). One control bound to rabbit pyruvate kinase (DYT1 dystonia). Figure 8.9 shows the positive binding in patients and the positive control (commercial anti-PYK antibody).

Table 8.a. Anti-rabbit pyruvate kinase antibodies in patients compared to controls.

Group	Positive binding (%)
Patients (n=20)	13 of 20 (65%)
Controls (n=20)	1 of 20 (5%)

However, at least one patient with strong antibodies against rat PYK did not react with rabbit PYK, adding doubt to the usefulness of rabbit PYK as a surrogate autoantigen.

8.9.3. Conclusion

Although rabbit PYK is a useful screen, the presence of a probable false negative result suggests that rabbit PYK may not be a perfect surrogate autoantigen. It is likely that recombinant human PYK M1 would be required for definitive assays. It was not possible to make recombinant human PYK M1 during the time constraints of this thesis.

Chapter 9. In vitro pathogenic effects of anti-neuronal antibodies on neuronal function

9.1. Possible pathological function of antibodies on neuronal function

The glycolytic enzymes non-neuronal and neuronal enolase, aldolase C and pyruvate kinase M1 all reside in the cytoplasm of neurones. However, as discussed later, the enzymes also reside on the neuronal membrane. The enzymes are multifunctional proteins, and are thought to have a role on the membrane in energy metabolism, neuronal cell well-being and neurotransmission support (discussed in detail later).

Clearly, it is not possible to examine the potential pathogenic role of these antibodies in this thesis in detail. However, I was able to examine two possible functional effects of antibodies on neuronal function:

- Effect of anti-NNE antibodies on the function of NNE as a plasminogen receptor on the neuronal membrane.
- Effect of commercial antibodies (anti-enolase, anti-aldolase, anti-pyruvate kinase) on neuronal survival (measuring apoptosis). This was performed by Dr Jenny Pocock at the cell signalling lab, Institute of Neurology.

9.2. Do the anti-NNE antibodies have functional effects on non-neuronal enolase as a plasminogen receptor?

9.2.1. Aim and background

Non-neuronal enolase acts as a plasminogen receptor on the neuronal surface, and mediates interaction with dopaminergic neurones (Nakajima K et al., 1994). The streptococcal enolase also acts as a plasminogen receptor, and is involved in its ability to invade tissue. There are previously described methods examining the interaction between plasminogen and enolase. I therefore followed these methods, with some modifications (Moscato S et al., 2000; Pancholi V and Fischetti VA, 1998).

9.2.2. Method

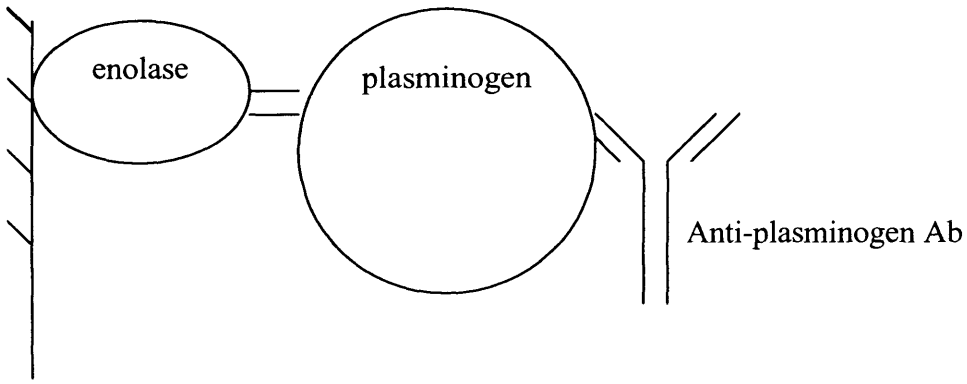
In the first instance, I wanted to demonstrate that plasminogen binds to NNE. This is obviously essential if I am to assess the effects that antibodies may have on this interaction. I therefore purchased human plasminogen and incubated the plasminogen with commercial human NNE that had been electrophoresed and transferred to nitrocellulose. I used an anti-plasminogen antibody as my detection. To control this experiment, I did one blot without pre-incubation with plasminogen to see if there was plasminogen resident in the antigen preparation.

1. The protein (human NNE or rat brain fraction containing NNE) was first electrophoresed, transferred to nitrocellulose and blocked.
2. Once blocked, the blots were incubated overnight with 20 μg human plasminogen in 20 ml normal saline. In accordance with the method published by Pancholi, 2mmol of PMSF (Phenylmethylsulfanylfluoride) was also added to the incubation solution (Pancholi V and Fischetti VA, 1998).
3. After incubation, the blots were washed for 1.5 hours with 0.025%Tween with changes of wash solution every 10 minutes.

4. After washing, the blots were incubated with anti-plasminogen antibody (HRP conjugated) 1:10,000 dilution for 2 hours.
5. The blots were washed as before for 2 hours.
6. The blots were then developed using ECL.

Lane	1	2	3
Antigen	Rat brain fraction (45-65%) 30µl	Human NNE 3µg	Rat brain fraction (45-65%) 30 µl
Plasminogen incubation	20 µg	20 µg	None
Anti-plasminogen Ab	+	+	+

Figure 9.1. Schematic representation of plasminogen-enolase experiment.



9.2.3. Results

Using this method, plasminogen binds to human NNE (Figure 9.2). Plasminogen also binds to a 45 kDa protein in the rat brain antigen (presumably rat NNE). In addition, there is strong binding to a protein in the rat brain tissue at 38 kDa. It is possible that this represents glutaraldehyde 3-phosphate dehydrogenase, the other recognised plasminogen receptor. In the blot that was not pre-incubated with plasminogen, there was no antibody reactivity in the rat tissue. This demonstrates that the reactivity in the

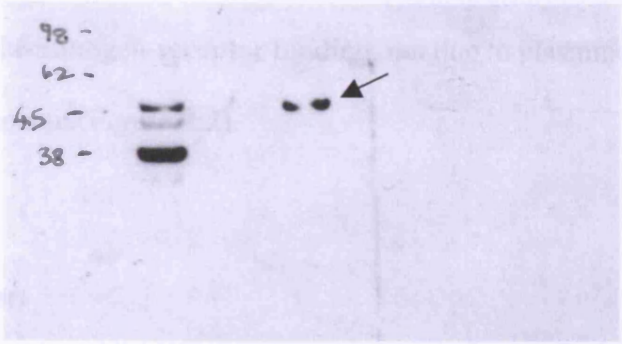


Figure 9.2. Plasminogen binding to human NNE (lane 2, arrowed), and to a similar 45 kDa protein (presumed rat NNE) and 38 kDa protein (possible GDH) in rat brain (lane 1). There was no anti-plasminogen antibody binding to the rat brain tissue that had not been pre-incubated with plasminogen (lane 3).

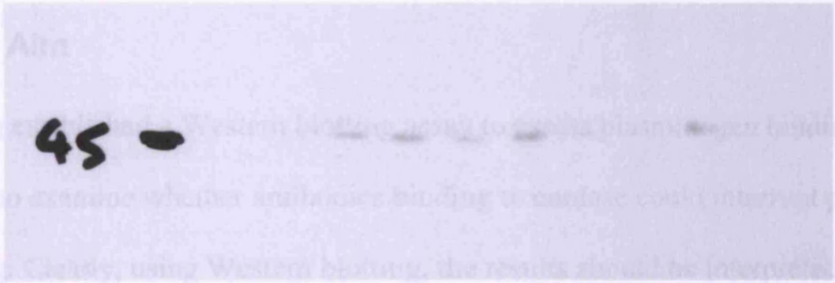


Figure 9.3. Inhibition of plasminogen binding to enolase by patient serum. Patient serum inhibits plasminogen binding to enolase (lanes 4 and 6), but the control serum (lane 7) also inhibits plasminogen binding.

Figure 9.4. Patient EW IgG pre-incubation with human NNE (lanes 5-8) has no effect on plasminogen-enolase binding (control in lane 4).

rat tissue is due to plasminogen-receptor binding, not due to plasminogen resident in the rat tissue homogenate (Figure 9.2).

9.2.4. Conclusion

Using Western blotting, it is possible to bind plasminogen to enolase, and demonstrate this reaction using anti-plasminogen antibody.

9.3. Human and commercial antibody effect on plasminogen binding to non-neuronal enolase

9.3.1. Aim

Having established a Western blotting assay to assess plasminogen binding, I next aimed to examine whether antibodies binding to enolase could interrupt plasminogen binding. Clearly, using Western blotting, the results should be interpreted as provisional, as the SDS preparation alters proteins. Western blotting is not a physiological system. It would be preferable to use proteins in their physiological state.

9.3.2. Method

1. Commercial human NNE was used as the antigen substrate. 5 μg was diluted in SDS, DDT and double distilled water and loaded onto 2D 4-12% Bis-Tris gel. The gel was electrophoresed using the standard method, transferred to nitrocellulose and blocked as per standard Western blotting protocol.
2. The primary antibody (patient serum or control polyclonal anti-enolase antibody) was diluted as stated below and incubated overnight.

3. The blot was washed in 0.05% Tween normal saline solution for 1.5 hours with changes of wash solution every 10 minutes.
4. 20 µg plasminogen was diluted in 20ml normal saline plus 2mmol PMSF, and incubated for 2 hours.
5. The blot (in manifold) was washed as above.
6. The secondary antibody (anti-plasminogen) was loaded 1:5000 as stated in the table for 2 hours.
7. The blot was washed as stated above and developed using ECL.

Lane	Primary antibody	Plasminogen incubation	Secondary antibody
1	-	-	Anti-plasminogen HRP 1:5000
2	Anti-enolase (C-19) 1:1000	-	Anti-goat HRP 1:5000
3	-	+	Anti-plasminogen HRP 1:5000
4	GdS serum 1:150	+	Anti-plasminogen HRP 1:5000
5	GdS serum 1:500	+	Anti-plasminogen HRP 1:5000
6	EW serum 1:150	+	Anti-plasminogen HRP 1:5000
7	AC 49 serum 1:150	+	Anti-plasminogen HRP 1:5000
8	-	+	Anti-plasminogen HRP 1:5000

9.3.3. Results

The anti-enolase antibody (lane 2) and plasminogen incubation (lane 3) confirmed that human NNE binds plasminogen (Figure 9.3). The patient serum inhibited plasminogen binding in patients (lanes 4 and 6), but also in the healthy control (lane 7). This healthy control does not have anti-NNE antibodies on previous evaluation.

9.3.4. Conclusion

Although the patient serum appeared to interfere with plasminogen binding, the control produced the same interference. I therefore wanted to check whether it was IgG that was interfering with enolase-plasminogen binding. It is possible that other components in the serum (complement or drugs, etc.) are interfering with the enolase-plasminogen interaction, specifically or otherwise. I therefore needed to purify IgG and de-complement the serum, and re-test this experiment.

9.4. Plasminogen binding to enolase: interruption by patient IgG?

9.4.1. Aim

I therefore aimed to re-test this experiment, this time with patient IgG purified from a patient with previously demonstrated anti-human NNE antibodies.

9.4.2. Method

The patient IgG was purified using the method described in Protein A IgG purification (chapter 5). The IgG concentration was measured using the Biuret method. The IgG fraction contained 1.4 μg/μl. The antigen (NNE loaded onto nitrocellulose) and method was the same as previously described in section 9.3. The manifold was loaded as stated below:

Lane	Primary antibody	Plasminogen incubation	Secondary antibody
1	-	-	-
2	Anti-enolase (C-19) 1:1000	-	Anti-goat HRP 1:5000
3	EW 100µl IgG	-	Anti-human HRP 1:5000
4	-	+	Anti-plasminogen HRP 1:5000
5	EW 250µl IgG	+	Anti-plasminogen HRP 1:5000
6	EW 25µl IgG	+	Anti-plasminogen HRP 1:5000
7	EW 2.5µl IgG	+	Anti-plasminogen HRP 1:5000
8	EW 0.25µl IgG	+	Anti-plasminogen HRP 1:5000

As can be seen, this experiment aimed to compare the plasminogen binding to human NNE with varying amounts of patient IgG pre-incubated with the human NNE. Lane 5 contained a large amount of IgG (350 µg), almost certainly in excess of the available human NNE on the blot (5µg on the whole 2D gel).

9.4.3. Results

As can be seen (Figure 9.4), the patient IgG reacts with human NNE (lane 3), although the background is very high, probably due to overloading the well with IgG. Lane 4 is the control, which had no pre-incubation of IgG before incubation with plasminogen. As can be seen, lanes 5-8 had decreasing concentrations of patient IgG pre-incubated with human NNE, before plasminogen incubation. There is no determinable alteration of plasminogen binding with NNE secondary to IgG preincubation. This experiment, suggests that patient anti-NNE antibodies do not affect enolase-plasminogen binding under these experimental conditions (discussed later).

9.4.4. Conclusion

The patient IgG chosen (EW) with anti-human NNE antibodies had no effect on plasminogen-enolase binding. The serum did interfere with this binding, suggesting that other serum constitutents, such as complement or drugs, may interfere with this interaction. However, under these experimental conditions, patient IgG alone did not have any effect. The conclusions are therefore:

1. Anti-human NNE IgG 1, 2 or 4 antibodies do not effect enolase-plasminogen binding.
2. In some patients and controls, a serum constituent other than IgG 1, 2 or 4 inhibits enolase-plasminogen binding.

9.5. Anti-neuronal glycolytic enzyme antibody effects on neuronal apoptosis

9.5.1. Introduction

I next aimed to determine whether the antibodies had effects on neuronal function. Although non-specific, I used apoptosis as a measure of the effects of antibodies on neuronal function. These experiments were performed by Dr Jenny Pocock, Institute of Neurology. In these provisional experiments, rather than using patient purified polyclonal IgG, I used commercial antibodies against enolase, aldolase and pyruvate kinase.

9.5.2. Cerebellar granule cell cultures

Rat cerebellar neurones were cultured and used as the neuronal cell line. Primary cultures of cerebellar granule cells (CGCs) were isolated from 3-4 day old Wistar rat pups and prepared as previously described (Evans JO and Pocock JM, 1999). Briefly, the rat pups were killed by cervical dislocation followed by decapitation in accordance with the Scientific Procedures Act, UK, 1986. Following mechanical and enzymatic isolation, the neurones were plated on 13 mm poly-D-lysine coated coverslips at a density of 0.7 x 10^6 cells per coverslip and maintained in minimum essential medium with Earle's salts supplemented with 25 mM KCl, 30 mM glucose, 25 mM $NaHCO_3$, 1 mM glutamine, 10 % foetal calf serum, and 50 U/ml penicillin, 50 µg/ml streptomycin and 6 µg/ml ampicillin. After 24 h *in vitro*, 20 µM cytosine arabinoside (1-β-D-arabinofuranosylcytosine, AraC) was added to prevent the proliferation of non-neuronal cells. The cultures were maintained at 37 °C in 5 % CO_2. Cells were used after 7 days *in vitro* (DIV).

9.5.3. Treatment of cells with commercial antibodies.

10µg/ml of commercial antibody was incubated for 24 hours. Control cells had the same volume of distilled water added alone. A control antibody (anti-Hu) was used in the same concentration.

9.5.4. Assessment of cellular apoptosis

The number of apoptotic cells was assessed by Hoechst 33342 (bisbenzimide trihydrochloride) staining (Kingham PJ et al., 1999). Cells were fixed in phosphate buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 2.9 mM $NaH_2PO_4.2H_2O$, 25.8

mM Na_2HPO_4) with 8 % formaldehyde for 15 min at 4 °C, and then stained with 5 mg/ml Hoechst 33342 for 15 min. The morphology of the nucleus was viewed using a fluorescence microscope with excitation at 365 nm and emission at > 490nm. The number of cells with pyknotic, brightly stained nuclei and those with normal, dull-staining nuclei were scored in 10 fields of view per coverslip, and recorded as a percentage of the total cells. Each commercial IgG sample was tested on at least 2-3 coverslips.

9.5.5. Results of antibody effects on apoptosis

The extent of neuronal apoptosis induced by the commercial antibodies (enolase, pyruvate kinase, aldolase) was compared with the control commercial antibody (HuD) and the control blank (Table 9.a also represented in Figure 9.5). Anti-enolase, anti-pyruvate kinase and anti-aldolase antibodies all induced a significantly higher rate of apoptosis in the cultured neurones compared to the blank control (all $p<0.0005$) and the anti-HuD ($p<0.01$).

Table 9.a. Percentage of apoptotic cells after incubation with commercial antibodies.

Antibody	Apoptosis % cells (+/- SEM)
Blank	10.6 +/- 0.94
HuD	15.6 +/- 0.83
Enolase	32.6 +/- 1.65
Pyruvate kinase	27.2 +/- 2.2
Aldolase	27.3 +/- 1.6

Figure 9.5. Percentage of apoptotic cells after incubation with commercial antibodies (and SEM). 1: Blank. 2: Anti-HuD antibodies. 3: Anti-Enolase antibodies. 4: Anti-Pyruvate kinase antibodies. 5: Anti-Aldolase antibodies.

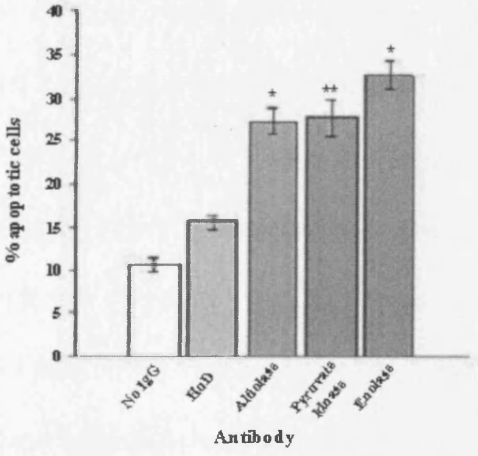


Fig: *In vitro* induction of apoptosis of cerebellar granule cells. Anti-aldolase, pyruvate kinase and enolase antibodies all resulted in a significant increase in the number of apoptoctic cells compared to no IgG. * $p < 0.0001$, ** $p = 0.0003$. There was no significant increase in apoptosis with anti-HuD antibodies.

9.6. Conclusions of antibody effects on neuronal function.

In conclusion, patient human anti-enolase antibodies do not appear to alter enolase activity as a plasminogen receptor. In contrast, the commercial antibodies against neuronal glycolytic enzymes induce apoptosis compared to controls, suggesting that antibodies against these autoantigens potentially have pathogenic function. These experiments will need to be repeated using purified human IgG specific to the

autoantigens. In addition, more detailed pathogenic effects on neuronal function will be required, beyond the less specific markers we have measured so far (apoptosis).

Chapter 10. Discussion of clinical and immunological thesis findings

10.1. Spectrum of post-streptococcal neuropsychiatric phenotypes

10.1.1. Post-streptococcal dyskinesias

I presented the experience of paediatric post-streptococcal dyskinetic movement disorders in a tertiary care setting. As this cohort was derived from a referred sample and not epidemiological in design, the findings cannot truly represent a community sample. It is possible that the tic phenotype (PANDAS) is under-represented, as many of these patients will be treated in the community and not referred to a hospital. In contrast, patients with chorea, dystonia and Parkinsonism are very likely to be referred for secondary and tertiary paediatric care. Also there may be referral bias within the tic cohort, as patients with impairing tics are more likely to be referred for opinion. Likewise, patients with associated psychiatric comorbidity are also more likely to be referred to tertiary care.

However, despite these limitations, this cohort highlights important findings. Specifically, the spectrum of post-streptococcal dyskinesias is not restricted to chorea, but includes motor tics and dystonia. This cohort, now published (Dale RC et al., 2004), provides further support for the existence of the PANDAS phenotype, although PANDAS will remain a controversial diagnosis until more robust diagnostic assays become available (Kurlan R, 2004). It is perhaps not surprising that a variety of movement disorders may occur in the context of post-streptococcal neurological disease. Rarely do basal ganglia syndromes result in only one extrapyramidal phenotype; indeed a variety of extrapyramidal movement disorders are reported in 'basal ganglia disorders' such as Huntington's disease and Wilson's disease.

By analysing differences between the chorea and tic subgroup, I attempted to determine what variables might dictate phenotypic expression. Although the patient age did not clearly influence the dyskinesia phenotype, the sex distribution demonstrated male predominance in the tic cohort and female predominance in the chorea subgroup. As discussed in the introduction, this sex distribution has been previously reported in Sydenham's chorea after puberty (Nausieda PA et al., 1980) and tic disorders (Singer HS and Walkup JT, 1991), and may suggest an influence of sex hormones on phenotypic expression. The fact that oestrogen can precipitate chorea (chorea gravidarum and oral contraceptive pill) supports the possible role of oestrogen in the chorea phenotype (Nausieda PA et al., 1979). In this cohort, it was notable that there was a modestly elevated prevalence of neuropsychiatric disorders (particularly ADHD) preceding movement disorder onset, a finding previously reported by Mercadante (Mercadante MT et al., 2000). It is possible that ADHD could represent a specific neurodevelopmental risk factor for the later development of post-streptococcal movement disorders. Alternatively, the preceding psychiatric symptoms may be a consequence of previously unrecognised episodes of post-streptococcal autoimmunity. Only longitudinal and epidemiological studies would address these alternate hypotheses. It is also possible that the movement disorder phenotype is partly related to the particular cortico-striatal tracts involved in disease pathogenesis (Albin RL et al., 1989).

Vocal tics were common in the motor tic subgroup, however they were not exclusive to this group. Indeed, vocal tics are an unusual but previously recognised feature of Sydenham's chorea (Mercadante MT et al., 1997). Other than extrapyramidal movements, additional neurological features were uncommon although sleep disturbance (particularly insomnia) occurred in a significant proportion. It could be argued that the sleep disturbance is due to the disruption of normal sleep patterns by

extra movements. Alternatively, the aberrant neurochemistry producing the movement disorder could also affect sleep pathways. Recent reports of sleep disturbance in Parkinson's disease and Huntington's disease support this putative hypothesis (Silvestri R et al., 1995; Rye DB and Jankovic J, 2002). The association of post-infectious movement disorders with sleep disorders will also be discussed in the Parkinsonism/ encephalitis lethargica section.

Emotional and behavioural alteration was a common accompanying feature of the acute disease, regardless of the movement phenotype. The acute behavioural changes were often dramatic and rapid. Indeed, Swedo felt that the 'over-night' change in behaviour was characteristic of PANDAS, and often occurs before the chorea in SC (Swedo SE et al., 1993; Swedo SE et al., 1998). Indeed a number of the patients were admitted to hospital, such was the dramatic and surprising alteration in the child's behaviour. Frequently, the children suffered a 'change in personality', and became emotionally labile or aggressive. After the acute phase, formal interview demonstrated a high prevalence of ICD-10 psychiatric diagnoses. As demonstrated in previous cohorts of SC and PANDAS (Swedo SE et al., 1998; Mercadante MT et al., 2000), emotional disorders (particularly obsessive-compulsive disorder and anxiety disorders) were the most common, and the incidence of obsessive-compulsive disorder (OCD) was more common in chronic or relapsing SC (Asbahr FR et al., 1999). Indeed, OCD in this cohort was limited to the patients whose movement disorder had been present for more than 1 year. The shorter mean duration of illness in the SC cohort may be responsible for the reduced incidence of OCD in this subgroup. Otherwise, there were no clear differences in the psychiatric morbidity between the chorea and tic subgroups in this study. In addition to emotional disorders, conduct disorders and hyperkinetic disorders (such as ADHD) were also common. By contrast, psychotic symptoms were not seen in this cohort, in keeping with previous

reports (Swedo SE et al., 1993; Swedo SE et al., 1998). It would be of interest to perform follow-up assessments on this cohort to see if the psychiatric features resolve or remain (as found by Freeman in SC, Freeman JM et al., 1965).

The methodology used to determine psychiatric morbidity in this thesis is criticisable for a number of reasons. Firstly, the DAWBA was developed primarily for epidemiological studies rather than cohort studies. Although the DAWBA was validated by Robert Goodman during development and showed good correlation with conventional psychiatric methods, it would have been valuable to perform similar reliability checks during the psychiatric assessments included in this thesis. In addition, although psychiatric diagnoses are made by the DAWBA, there is little information regarding symptom profiles. Other interviews (such as CY-BOCS for OCD) would be required for more in-depth profiling.

The main difference between the chorea and tic subgroups was the presence of systemic features (carditis and arthritis) that were exclusive to the chorea subgroup. The cause of this important difference is unknown although it must be acknowledged that systematic cardiac examination in PANDAS patients was not performed in this case series. The recent report of echocardiographic abnormalities in PANDAS patients demonstrating mitral valve changes emphasises the need for careful investigation for asymptomatic cardiac disease in all post-streptococcal neuropsychiatric patients in the future (Cardona F et al., 2003).

Although some patients within this cohort have had transient disease or resolution of the movement disorder within a year, a large proportion of patients have had persistent or relapsing disease. The high proportion of patients with protracted disease may be due to selection bias, as patients with milder transient disease are unlikely to be referred to a tertiary referral centre. Only epidemiological studies could characterise the true incidence of chronic and persistent disease. As discussed in the

introduction, previous studies suggest that Sydenham's chorea becomes persistent in 20-50% of patients (Nausieda PA et al., 1980; Cardoso F et al., 1999). Swedo's proposed diagnostic criteria for PANDAS require two or more exacerbations of motor tics or OCD with streptococcal infections (Swedo SE et al., 1998). If we believe that Sydenham's chorea and PANDAS are biologically similar disorders, Swedo's clinical criteria of PANDAS would exclude a significant proportion of PANDAS patients with monophasic disorders. Laboratory confirmation of recent GAS infection is necessary for a diagnosis of post-streptococcal dyskinesia, however positive GAS serology is common in paediatric populations and is unlikely to be a specific diagnostic tool used in isolation. Indeed, 18% of the neurological controls in this thesis cohort had elevated streptococcal serology. Previous childhood control groups in the US have even higher rates of positive streptococcal serology (Singer HS et al., 1998). This has consequently led to diagnostic difficulty, and even questioned whether the 'PANDAS' phenotype exists (critically reviewed in Singer HS and Loiselle C, 2003). This emphasises the need for improved clinical and laboratory criteria for the diagnosis of PANDAS. Other investigation was usually normal, and MR imaging revealed no abnormalities in the majority.

The prevalence of movement and emotional disorders in first-degree family relatives suggests that a genetic predisposition is important in disease development, in addition to the environmental trigger (Lougee L et al., 2000). It could be argued that the high incidence of neuropsychiatric disease in 1st degree family members is due to a genetically determined neurodevelopmental vulnerability. Alternatively, the psychiatric disease in parents and siblings could have had a psychological impact on the patients. My more favoured hypothesis is that disease is related to a genetically determined autoimmune predisposition. The positive family history of post-streptococcal autoimmune syndromes (Sydenham's chorea, PANDAS and rheumatic

fever) in this study and previous cohorts of SC supports this genetic immune hypothesis (Nausieda PA et al., 1980). Whether the genetic immune vulnerability is related to the B-lymphocyte marker D8/17 (as discussed in the introduction), or a hitherto unrecognised genetic vulnerability remains unknown. Finally, it is also possible that genetic vulnerability is mediated via a combination of these mechanisms (neurochemical, immunological and psychological).

In conclusion, a broad spectrum of dyskinetic extrapyramidal movements and neuropsychiatric disorders may occur after GAS infection. Although the clinical similarities between the chorea and tic subgroups suggest that SC and PANDAS may be two phenotypes of the same immune mediated basal ganglia disorder, other authors have highlighted the differences (Murphy TK et al., 2000). Phenotypic expression may depend on other variables including the specific cortico-striatal circuits involved, developmental status, genetic predisposition and patient's sex. Many of the PANDAS patients fulfilled diagnostic criteria for Tourette's syndrome. Improved understanding of the disease mechanism in post-streptococcal neuropsychiatric disease could significantly improve our knowledge of the neurochemistry and neuroanatomy of common childhood diseases.

10.1.2. Post-streptococcal acute disseminated encephalomyelitis

The clinical features in the 10 patients with post-streptococcal acute disseminated encephalomyelitis (PSADEM) were remarkable due to the high incidence of dystonia and rigidity. In contrast, extrapyramidal movement disorders are rarely reported in previously described ADEM cohorts (Dale RC et al., 2000; Tenembaum S et al., 2002). No patient had chorea or tics, clinically differentiating the PSADEM patients from SC or PANDAS. Seventy percent of the patients had a behavioural alteration characterised by emotional lability, inappropriate laughter, separation anxiety and confusion. The behavioural alteration was similar to that seen in the acute stages of SC and PANDAS. In addition to the movement disorder and behavioural change, most patients also presented with encephalopathy requiring rapid assessment and management. Despite the dramatic presentation, the majority of the patients made a good recovery, although one patient has obsessive-compulsive disorder, and another has persistent hemidystonia. The good outcome may be related to the natural course of the disease, or could be due to the prompt use of immunosuppressive treatments in these cases (intravenous methylprednisolone). These children were given immunosuppressive treatments in the acute or hyperacute stages of disease, because of the encephalopathic presentation requiring prompt assessment, diagnosis and treatment. In contrast, patients with SC and PANDAS rarely achieve such prompt management. If prompter diagnoses and treatment could occur in SC and PANDAS patients, it is tempting to speculate that these children could have better outcomes. Interestingly as seen in SC and PANDAS, relapses may occur. In the two PSADEM cases that relapsed, the relapses were related to further streptococcal infections.

The most important differentiating feature of PSADEM from SC and PANDAS (other than the encephalopathic clinical features) is the presence of an inflammatory CSF and the presence of inflammatory lesions on T2-weighted MR imaging. The inflammatory lesions on MR imaging were most prevalent in the basal ganglia regions, although there were frequently disseminated lesions throughout the CNS. These MR imaging findings provide important information into the pathogenesis of post-streptococcal neuropsychiatric disease. Although the basal ganglia appear to be particularly vulnerable to post-streptococcal immune mediated attack, disease also occurs (at a lower level) in other parts of the CNS. This imaging finding in PSADEM is supported by the pathological reports of SC (described in the introduction): Although the basal ganglia is often the most floridly involved brain region, other parts of the CNS (particularly the cortex) are pathologically involved. The presence of CSF abnormalities in some patients with PSADEM (in contrast to SC and PANDAS), also suggests that the inflammatory reaction is more developed in PSADEM than in SC or PANDAS.

In conclusion, PSADEM has a number of similarities with SC and PANDAS (the presence of an extrapyramidal movement disorder, behavioural alteration and relapsing remitting nature). However, PSADEM cases frequently have more disseminated CNS involvement (clinically and radiologically) suggesting that, although the basal ganglia is the most vulnerable region, the immune attack can generalise to the rest of the CNS.

10.1.3. Post-streptococcal Parkinsonism and encephalitis lethargica

The index cases reported in this thesis had remarkable similarity to the historical reports of encephalitis lethargica (EL). This mysterious disease was last seen in epidemic forms in the 1920's, and was described in detail by von Economo (von Economo, 1931). The central features of the disease were sleep disorder, lethargy, extrapyramidal movements (Parkinsonism and dyskinesias) and neuropsychiatric disturbance (obsessive-compulsive disorder, catatonia, mutism, apathy and conduct disorders). The neuropsychiatric phenomenology of EL led to notions that the deep grey matter (particularly basal ganglia) may be involved in the control of mood, emotion, behaviour and volition (Cheyette SR and Cummings JL, 1995; Ward CD, 2003). Furthermore, von Economo used EL as a model of sleep disturbance, and his anatomical localisation of sleep control to the midbrain and deep grey matter has been confirmed by contemporary studies (Saper CB *et al.*, 2001). The mortality of epidemic EL was between 20-40%. Of the survivors, many were left with Parkinsonism, dyskinesias or psychiatric disease. The cases described in this series had similar characteristics to those described by von Economo. In this series, one of the patients died, and five had reduced conscious level and required ventilation. It is probable that these ventilated patients would have died were it not for contemporary intensive care. A complete recovery occurred in a minority of patients in this series, the majority are still suffering continued psychiatric and movement disorders. The course of disease was monophasic or fluctuating, unlike the progressive course characteristic of the metabolic, biochemical or inherited causes of Parkinsonism. The cerebrospinal fluid was abnormal in about 50% of epidemic EL cases, mild elevation of protein and mild lymphocytosis being characteristic (von Economo, 1931; McCall S *et al.*, 2001). The cerebrospinal fluid abnormalities found in the

patients in this report are similar to recent cases of sporadic EL (Howard RS and Lees AJ, 1987; Blunt ST *et al.*, 1997; Kiley M and Esiri MM, 2001), and the presence of intrathecal synthesis of oligoclonal bands have been proposed to be a useful marker of disease (Williams A *et al.*, 1979; Howard RS and Lees AJ, 1987). Either intrathecal or mirrored pattern of OCB was seen in 69% of the patients described in this report. By contrast, all CSF PCR studies were negative, making neurotropic viral encephalitis unlikely. When considered together, oligoclonal bands, CSF pleocytosis and elevated CSF protein were abnormal in 81% of patients, and although in keeping with an immune mediated pathogenesis, are not specific to this phenotype. The MR neuroimaging in this cohort was abnormal in 40%; characteristic features were increased signal in the basal ganglia, substantia nigra and tegmentum, features recently proposed to be suggestive of EL (Verscheren H and Crols R, 2001). There were some similarities with the EL imaging abnormalities to those seen in PSADEM, which further suggests that PSADEM and EL may lie on the same disease spectrum. The enhancement resolved after the acute stage in the few patients who had convalescent imaging.

The pathological features of both epidemic EL and contemporary EL have shown perivascular lymphocytic (plasma cell) cuffing, which predominantly involved the midbrain and basal ganglia (von Economo, 1931; Rail D *et al.*, 1981; Kiley M and Esiri MM, 2001). A recent post-mortem case of a patient with the EL phenotype demonstrated an unexpected excess of perivascular plasma cells, which were distended by IgG. The authors concluded that a brisk humoral response was occurring (Kiley M and Esiri MM, 2001). In the one post-mortem case, we found similar histopathological findings with perivenous lymphocytic cuffing predominantly of the basal ganglia, although there was lymphocytic infiltration seen elsewhere in the brain at a lower level. The lymphocytes were both T and mature B lymphocytes. Other than

secondary reactive astrocytes and macrophage activation, there were no other striking pathological features. Of note, there were no viral inclusions. Interestingly, reports during the 1920's described remarkable pathological similarity between encephalitis lethargica and Sydenham's chorea (Greenfield JG and Wolfsohn JM, 1922). Chronic changes included neuronal loss and neurofibrillary degeneration in the midbrain and basal ganglia. These pathological findings again suggest that, although the basal ganglia appears to be particularly vulnerable to this encephalitic process, the disease is not confined to the basal ganglia only, and occurs at a lower level elsewhere in the CNS.

The precipitating micro-organism of epidemic EL remains unknown. Although EL has been associated with influenza (Ravenholt RT and Foege WH, 1982), von Economo suspected that an as yet unidentified virus or alternative process was responsible. Studies during the 1920's showed no clusters of disease suggesting an endemic presence as opposed to an imported epidemic (James SP, 1918). Unlike influenza, EL was not very contagious and even intra-familial spread was unusual (von Economo, 1931). Further evidence against influenza being the cause of EL was that von Economo had observed EL for 3 years before the onset of the great influenza pandemic. In addition, patients with EL rarely had influenza before neurological onset; in one early report of 76 EL cases, only 4 had influenza in the preceding 6 months (Stallybrass CO, 1923).

Recent PCR examination of five acute EL brains from the 1920's epidemic revealed no influenza RNA. Unlike respiratory tissue from patients who died of the 1918 influenza viral pneumonia, brain tissue from EL patients had no influenza RNA. The authors concluded that the 1918 influenza virus was unlikely to be directly responsible for the outbreak of EL (McCall S *et al.*, 2001). A separate recent study also demonstrated the lack of influenza genes in archived formalin-fixed brain

samples of EL patients from 1916 to 1920 (Lo KC *et al.*, 2003). Likewise contemporary EL cases have failed to demonstrate evidence of invasive encephalitis secondary to neurotropic viruses (Duvoisin RC and Yahr MD, 1965; Williams A *et al.*, 1979; Rail D *et al.*, 1981; Howard RS and Lees AJ, 1987; Elizan TS and Casals J, 1989; Blunt ST *et al.*, 1997; Kiley M and Esiri MM, 2001), although one case occurred as a post-infectious complication of mycoplasma infection (Al-Mateen M *et al.*, 1988). A recent review concluded that clinically, epidemiologically and morphologically, EL and the 1918 influenza virus represent distinct entities (Reid AH *et al.*, 2001).

Interestingly, during the epidemic attention had focused on a streptococcus as a possible etiological agent. A number of investigators had detected streptococci in the throats of patients suffering from EL (Wilson SAK, 1918; Harris W, 1918; von Economo, 1931). In addition, they were able to induce an encephalitis lethargica-like illness in dogs after vaccination with streptococcal organisms (von Economo, 1931). However, as gram positive organisms were never isolated from the brain, the authors concluded that it was unlikely to have been the primary etiological agent.

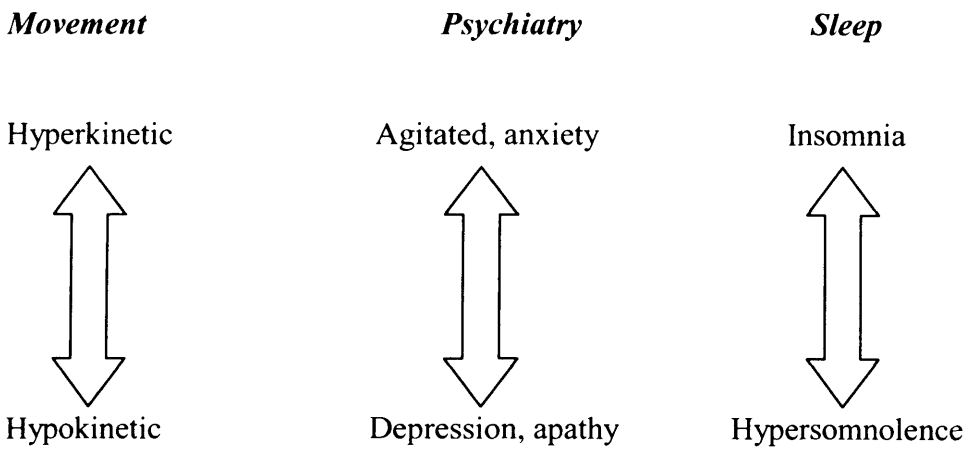
The hypothesis that the EL phenotype could be aetiologically similar to Sydenham's chorea is potentially appealing. Any hypothesis must be able to explain why the clinical, neuroimaging and pathological characteristics are relatively restricted to the deep grey matter. Invasive viral encephalitis rarely localizes so specifically, although Japanese B encephalitis is one notable exception. As pharyngitis was a common precedent of epidemic and contemporary EL, I examined the hypothesis that the EL patients described in this cohort were secondary to post-infectious autoimmunity directed against the deep grey matter. In this series, we were able to demonstrate recent streptococcal infection in significantly more EL patients compared to neurological and healthy control groups. It is therefore possible that streptococcus

may play an aetiological role in this EL phenotype, as it does in SC. However a proportion of the EL patients in this cohort had no evidence of preceding streptococcal infection. However, autoimmune complications commonly occur many weeks or months after the precipitating organism, and growth of the organism is rarely possible. For example, in Sydenham's chorea the ASOT is elevated in only 73% of patients due to the latent onset of neurological disease after streptococcal infection (Taranta A and Stollerman GH, 1956). I therefore propose that although the association with streptococcus is a useful line of further investigation, alternative environmental triggers (such as phylogenetically related micro-organisms) may be important in disease evolution of this EL phenotype.

10.1.4. Overview of the clinical spectrum of post-streptococcal neuropsychiatric syndromes

The clinical findings of this thesis are now presented in schematic form (Figure 10.1).

Figure 10.1. Schematic summary of the clinical spectrum of post-streptococcal neuropsychiatric disease.



10.2. Identification of autoantigens in post-streptococcal neuropsychiatric disease

10.2.1. Overview of candidate autoantigens

A summary of the autoantigens defined in this thesis are given in table 10.a.

Table 10.a. Summary of autoantigens defined in this thesis

Western blotting	Defined human autoantigen
40 kDa	Aldolase C
45 kDa lower band	Neuron-specific enolase
45 kDa upper band	Non-neuronal enolase
60 kDa	Pyruvate kinase M1
98 kDa	Neuron-specific enolase (dimer)

In summary, the autoantigens are all glycolytic enzymes. I will now discuss the characteristics of the autoantigens with relevance to their existence as potential autoantigens for autoantibody mediated attack.

10.2.2. Localisation of candidate auto-antigens

10.2.2.1 Isoforms (isoenzymes) of candidate autoantigens

10.2.2.1.1. Enolase

Enolase exists as two isoforms: the more acidic neuron-specific enolase (NSE) pI 4.7 and molecular weight 39.000 Daltons, and the more basic non-neuronal enolase (NNE) pI 5.9 and molecular weight 43,000 Daltons (Marangos PJ et al., 1978). NSE, also known as gamma enolase, exists primarily in neurons, wheras NNE, also known as alpha enolase, is ubiquitous in cells and exists primarily in glial cells in the brain

(although also exists on neuronal membrane, discussed later) (Marangos PJ et al., 1978).

The enzymes mainly exist as dimers with gamma gamma, alpha alpha, and alpha gamma forms (Marangos PJ et al., 1978; Keller A et al., 1994; Ueta H et al., 2004) although may exist as monomers on the neuronal surface (discussed later) (Nakajima K et al., 1994). NSE and NNE have 83% complete amino acid homology.

10.2.2.1.2. Aldolase C

Aldolase is also termed fructose 1,6 bisphosphate aldolase and exists as 3 isoforms A, B and C. Aldolase A is ubiquitous in all mammalian tissue, Aldolase B is predominantly found in liver, and Aldolase C is predominantly in brain (Buono P et al., 2001). Aldolase C is found at low levels in other tissues (about 2% of that in brain (Willson VJ and Thompson RJ, 1980). Aldolase C has a relative molecular mass of 39,000 Daltons and pI of 6.8 (Kukita A et al., 1988; Buono P et al., 2001). Aldolase A and C have 81% homology, wheras B and C have 70% homology (Kukita A et al., 1988).

10.2.2.1.3. Pyruvate kinase M1

Pyruvate kinase M1 has a molecular weight of approximately 60,000 Daltons, and a pI of 8.0. It often exists as a tetramer in the cytoplasm (Farrar G and Farrar WW, 1995). Broadly there are L and M isoforms. The M isoforms are further divided into M1, M2, M3 and M4 isoforms. M2 is the predominant form in spleen, leucocytes, lung and other systemic tissue. M1 is the predominant form in muscle (including heart) and brain (Marie J et al., 1976). Pyruvate kinase M1 and M2 have 99% homology.

10.2.2.1.4. Overview of isoforms

In summary, the candidate autoantigens are generally (with the exception of NNE) neuronal isoforms of the glycolytic enzymes enolase, aldolase and pyruvate kinase.

10.2.2.2. Localisation of candidate autoantigens

10.2.2.2.1. Overview of autoantigen localisation

Autoantibodies are considered more likely to be pathogenic if they bind to autoantigens that are present on a cell wall rather than intracellular. Although antibodies can gain access to a cell with an intact cell wall, this is generally considered unlikely and antibodies to intracellular and nuclear antigens are less likely to be pathogenic (Lang B et al., 2003).

10.2.2.2.2. Enolase

Over the last two decades, with the development of specific technologies for purifying different cell components, it has become possible to define cell membrane characteristics, with the aid of detergents such as Triton X-100 (to loosen the strong lipophilic membrane bonds (Lim L et al., 1983). It has been shown that glycolytic enzymes not only exist within the cell cytoplasm, but also on the cell membrane. Neuron-specific enolase and non-neuronal enolase exist on the plasma membrane of neurons (Lim L et al., 1983, Leung TK et al., 1987, Nakajima K et al., 1994). Enolase may reside as a monomer (Nakajima K et al., 1994) and/ or as a dimer (Ueta H et al., 2004). Indeed Ueta has demonstrated recently that the alpha gamma heterodimer exists in synaptic plasma membranes of neurons, making these autoantigens potentially available for autoantibody attack (Ueta H et al., 2004).

10.2.2.2.3. Aldolase C

Like NSE and NNE, Aldolase C has been shown to reside within the neuronal membrane and also the synaptic membrane (Builliard C et al., 1997; Bahler M et al., 1991). The amount of protein appears to be highest in the recycling vesicles (Builliard C et al., 1997).

10.2.2.2.4. Pyruvate kinase M1

Pyruvate kinase (PK) was demonstrated to reside on the neuronal membrane at the same time that enolase was revealed as a resident membrane protein (Leung TK et al., 1987; Lim L et al., 1983). Subsequently, the enzyme was shown to be tightly associated with the membrane, often residing in a complex (Dubinsky WP et al., 1998, discussed later), and may be enriched in nerve endings (Gali P et al., 1981). The pyruvate kinase exists at the cell surface in a membrane- anchored form (Hiebert SW et al., 1988). The anchor domain is a class II integral membrane protein (haemagglutinin/ neuraminidase) (Hiebert SW et al., 1988). Hiebert determined that neither the compete PK molecule nor its glycosylation was necessary for intracellular transport to the cell surface, nor expression at the surface (Hiebert SW et al., 1988).

10.2.2.3. Regional location of autoantigens within the brain

10.2.2.3.1. Overview

Most investigation to date has suggested that post-streptococcal autoimmune neuropsychiatric disease results in a CNS disorder relatively localised to the basal ganglia. I next wanted to investigate whether the defined autoantigens are present in the basal ganglia, specific to the basal ganglia or enriched in the basal ganglia.

10.2.2.3.2. Enolase

Non-neuronal enolase is present in neurons early in gestational life, but is replaced by neuron-specific enolase during gestational development (Gross J et al., 1990). Levels of NSE increase most rapidly in the phylogenetically old areas (midbrain, thalamus) compared to the new areas (cerebral cortex) (Marangos PJ et al., 1980, Gross J et al., 1990). NSE is present in neurons throughout the CNS, but at highest levels in the cerebral cortex, cerebellum, midbrain and basal ganglia (Jorgensen OS and Centervall G, 1982). There is no significant difference in the regional distribution of the dimeric alpha gamma enolase (Jorgensen OS and Centervall G, 1982). Regional comparison of NSE on neuronal membranes has not been done.

Non-neuronal enolase (NNE) is found in glia, but is also found on neuronal membranes (Nakajima K et al., 1994). Regional distribution of NNE on neuronal membranes has not been examined. In the mesostriatal system, there is a correlation between NSE expression (and cytochrome oxidase plus tyrosine hydroxylase) and the establishment of connections in the subpopulations of dopaminergic neurons (and their metabolic activity) (Silverman WF, 1992; Silverman WF, 1992).

10.2.2.3.3. Aldolase C

Aldolase C is present almost exclusively in neurons within the CNS, but also in neuroendocrine cells (Buono P et al., 2001, Thompson RJ et al., 1982, Inagaki H et al., 1988). Aldolase C mRNA is 4-fold higher in CNS neurons than astroglia (Popovici T et al., 1990). Aldolase C mRNA is predominantly found in the cerebellar neurons and to a lesser extent in medulla, striatum and occipital lobe (Buono P et al., 2001; Popovici T et al., 1990). The regional distribution of Aldolase C on the neuronal membrane has not been undertaken.

10.2.2.3.4. Pyruvate kinase

Pyruvate kinase M1 appears to be predominantly found in the cytoplasm of neuronal cell bodies, with scanty distribution in glia (Gali P et al., 1981). There is no apparent difference in distribution of pyruvate kinase in different neuronal populations of the cerebellum, cortex, brainstem or basal ganglia (Valenzuela A et al., 1987). Pyruvate kinase exists on the neuronal membrane (Lim L et al., 1983, Leung TK et al., 1987), although there has been no published study examining the regional distribution of pyruvate kinase on the neuronal membrane.

10.2.2.3.5. Conclusion

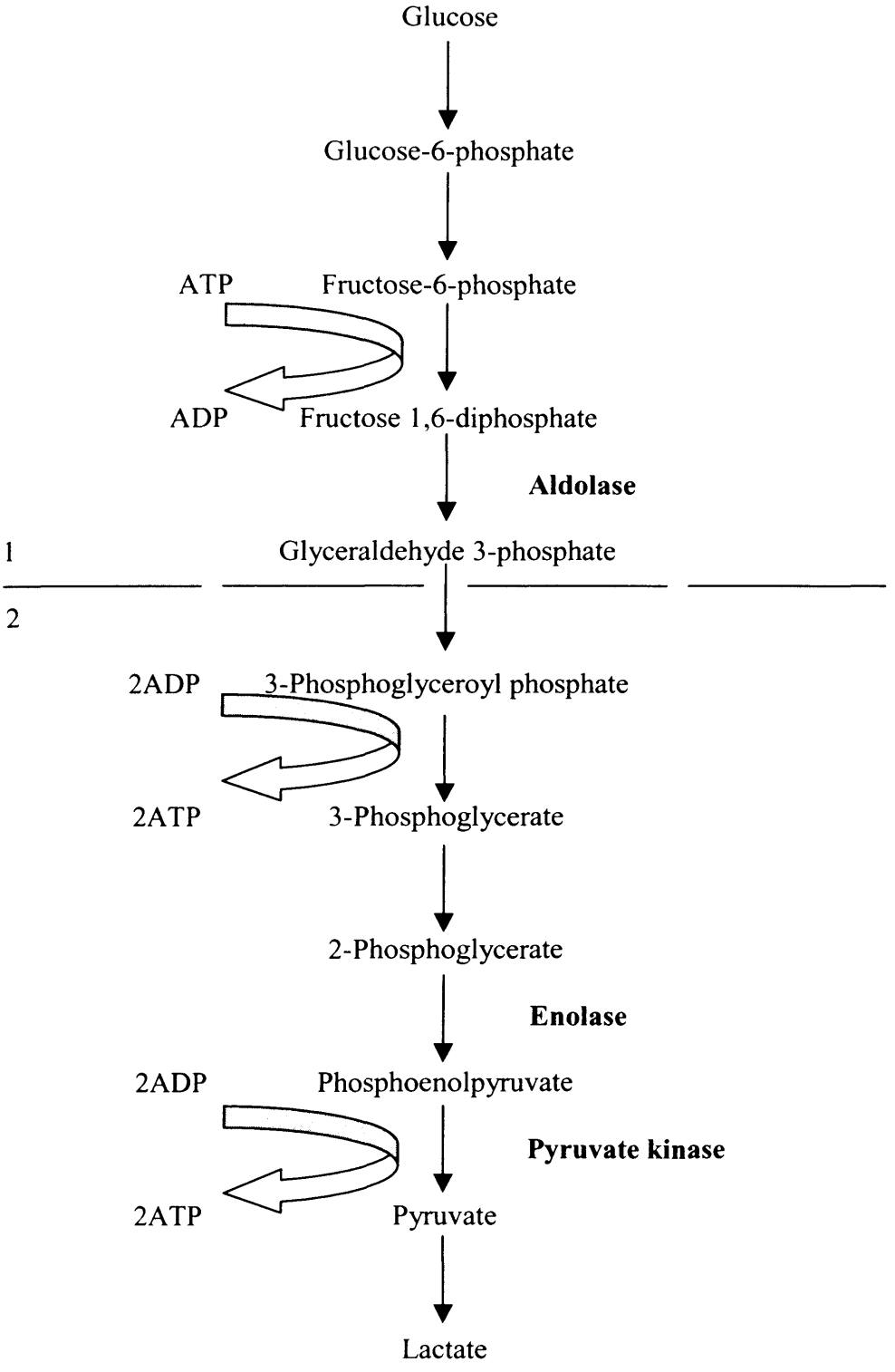
Although the autoantigens (apart from NNE) are enriched in, or specific to, neurons, there is no evidence to support basal ganglia specificity or enrichment of any of the autoantigens. There have been no studies of regional distribution of these proteins on the neuronal membrane.

10.2.3. Potential functional role of auto-antigens

10.2.3.1. Function of autoantigens as enzymes

All of the autoantigens are glycolytic enzymes and are centrally involved in energy metabolism in cells. The neuronal isoforms of these glycolytic enzymes are also involved in energy metabolism. The respective enzymes (and their enzymatic reaction) are described in Figure 10.2. As can be seen, the enzymes are centrally involved in the metabolism of ADP and ATP. As can be seen Aldolase is involved in the first stage of glycolysis, with input of priming ATPs. Both enolase and pyruvate kinase are involved in the second stage of glycolyis, with the conservation and production of energy as ATP.

Figure 10.2. Glycolysis and enzyme involvement.



10.2.3.2. Function of enolase on neuronal membrane.

The fact that enolase (both NNE and NSE) exist on the neuronal membrane suggest that these enzymes have specific roles at this site. The proteins derived from both synaptic plasma membranes and recycling vesicles do have enzymatic activity (Bulliard C et al., 1997). The specific activities of enolase (and also aldolase and pyruvate kinase) are elevated in the synaptosomal membranes suggesting that these enzymes (and other glycolytic enzymes) may provide the potential for enhanced glycolysis at these locations (Knull HR et al., 1985).

However, it has become clear that membrane surface expressed enolase has more roles other than those related to energy metabolism. Indeed enolase is now considered a multifunctional protein with a broader role in cell function (and therefore diseases) than previously considered (Pancholi V, 2001). It has been shown that NNE acts as a plasminogen receptor on neuronal membranes (Nakajima K et al., 1994). In addition, this plasminogen-enolase binding mediates interaction between microglia and dopaminergic neurons (Nakajima K et al., 1994). Interestingly, studies investigating plasminogen effects on mesencephalic neurons showed significant increases in tyrosine hydroxlase staining, but no alteration in gamma-aminobutyric acid (GABA) uptake or glutamic acid decarboxylase activity (Nagata K et al., 1993). These findings suggest that plasminogen results in a selective enhancement of dopaminergic activity in mesencephalic neurons (Nagata K et al., 1993). NSE is also known to form a tight complex with other glycolytic enzymes (including Aldolase C) and oxidoreductase complexes on neuronal membranes. The role of these complexes may include membrane fusion and protection of the neurone from oxidative stress during growth and development. In conclusion, enolase exists on the neurone membrane surface as a multifunctional protein with probable roles in energy metabolism, neurotransmission and cell well-being.

10.2.3.3. Function of aldolase on neuronal membrane

There has been less investigation regarding the role of aldolase on the neuronal membrane. However the protein does provide local membrane energy and is enzymatically active (Bulliard C et al., 1997). Adolase C forms an oxidoreductase complex with enolase and heat shock protein 70, TOAD64 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on the neuronal membrane (and synaptic membrane). Membrane Aldolase C in this complex is bound very tightly to GAPDH. This complex is thought to monitor oxidative stress at the cell surface and can induce the appropriate cellular response (Bulliard C et al., 1997).

In addition, experiments on other cell types have shown that aldolase binds tightly with ATPase protein pumps on the plasma membrane. It is thought that the aldolase-ATPase binding allows direct coupling of glycolysis to the proton pump (Lu M et al., 2001). It could therefore be speculated that aldolase and other glycolytic enzymes are closely involved in the energy provision and maintenance of ion channels on the neuronal membrane.

10.2.3.4. Function of pyruvate kinase on neuronal membrane

As with enolase and aldolase, pyruvate kinase is tightly bound to the neuronal membrane. Pyruvate kinase is a critical enzyme in the metabolism of ATP and ADP. Important experiments have shown that membrane glycolysis provides a preferential source of ATP for membrane function in order to maintain K^+ channels on myocytes (Weiss JN and Lamp ST, 1987), ATPase and calcium uptake on smooth muscle cells (Hardin CD et al., 1992) and Na^+ K^+ pumps on intestinal cells (Dubinsky WP et al., 1998). The maintenance of these pumps may be directly linked to functionally compartmentalised ATP to ADP ratios on the cell membrane (Dubinsky WP et al., 1998). Interestingly pyruvate kinase is also influenced by the female hormone oestradiol-17b. Administration of oestradiol-17b to rat brain neurons results in an

increase in soluble and membrane (synaptic) bound pyruvate kinase four hours after treatment (but not other glycolytic enzymes) (Kostanyan AA and Nazaryan KB 1991; Kostanyan A and Nazaryan K, 1992). These findings suggest that rat brain glycolysis regulation by oestradiol is carried out in neurons due to specific pyruvate kinase enzyme induction (Kostanyan A and Nazaryan K, 1992). This finding may provide a possible explanation for the increased vulnerability of females to the expression of chorea (chorea gravidarum and chorea on the oral contraceptive pill).

As a further example of the multifunctional roles of glycolyic enzymes, it has recently been shown that the monomer of pyruvate kinase acts as thyroid hormone (T3) binding protein. Binding of T3 to pyruvate kinase inhibits enzymatic activity, suggesting that this process may be centrally involved in the control of some cellular metabolic effects induced by thyroid hormones (Kato H et al., 1989). Perhaps it is therefore not surprising that hyperthyroidism is one of the causes of chorea.

10.2.4. Potential functional effects of auto-antibodies

10.2.4.1. Overview

It is theoretically possible that autoantibodies bound to membrane glycolytic enzymes can result in impairment of any of the following cellular functions: Energy metabolism, plasminogen binding, ion channel stability, neurotransmission and oxidative stress monitoring. Any of these functional effects are possible, or there may yet be undetermined impairments. It is possible that autoantibodies can directly and independently mediate functional effects, or functional effects may require secondary complement binding or even T-cell cytotoxicity.

10.2.4.2. Autoantibody effects on neuronal function

The experiments performed in this thesis failed to demonstrate impairments of plasminogen-enolase binding with commercial or human anti-enolase antibodies

alone. It is still possible that addition of complement may result in impairment of plasminogen binding and consequent neuronal dysfunction. The in vitro neuronal cell culture work presented in this thesis showed that commercial anti-neuronal glycolytic enzyme antibodies resulted in increased apoptosis. The mechanism of this cell death is unknown. Other groups have assessed the potential effects of antibodies against membrane glycolytic enzymes on cell function. A group have shown that anti-glycolytic enzyme antibodies are capable of impairing plasminogen binding to alpha enolase on leucocytes (Lopez-Alemany R et al., 2003). A separate group showed that the oxidoreductase activity of aldolase C/gamma enolase/TOAD64/ heat shock protein 70/ GAPDH complex on neuronal cells can be impaired by antibodies to the different components (Bulliard C et al., 1997). In addition, this effect was synergistic with sequential reductions in reductase activity with two or more antibodies. For example, anti-aldolase and anti-enolase antibodies reduced reductase activity on the neuronal membrane by 49% and 38% respectively, wheras using both anti-aldolase and anti-gamma enolase antibodies synergistically resulted in 78% reduction in reductase activity (Bulliard C et al., 1997).

10.2.5. Previous reports of auto-antibodies

There have been a number of previous descriptions of autoantibodies against enolase, aldolase and pyruvate kinase, particularly anti-alpha enolase antibodies (Pancholi V, 2001). Table 10.b. reviews the previous reports of autoantibodies against these glycolytic enzymes. Indeed enzymes are frequent targets of autoantibodies including:

- Anti-DNase 1 antibodies in systemic lupus erythematosus (Puccetti A et al., 1995)
- Anti-topoisomerase antibodies in systemic sclerosis (Douvas AS et al., 1979)

- Anti-myeloperoxidase antibodies in ANCA-positive vasculitis (Falk RJ et al., 1988)
- Anti-glucose-6-phosphate isomerase in Rheumatoid arthritis (Matsumoto I et al., 1999)

As can be seen, auto-antibodies against the ubiquitous forms of the glycoytic enzymes (alpha enolase specifically) have been found in other disorders although direct comparisons between the diseases is not possible as the methodologies differed. It is possible that some of the patient groups had lower, possibly less significant levels of autoantibodies. In addition, anti-aldolase A antibodies (low levels) have been found to be natural antibodies in healthy people (Pashov A et al., 2002). However, the most relevant previous report of anti-glycolytic enzyme antibodies is in post-streptococcal rheumatic fever (Fontan PA et al., 2000). This group demonstrated antibodies against alpha enolase in patients with rheumatic fever at significantly higher levels than healthy controls and controls with uncomplicated streptococcal pharyngitis. In addition, these antibodies showed cross-reactivity with the streptococcal surface enolase supporting a molecular mimicry hypothesis (discussed later, Fontan PA et al., 2000).

Table 10.b. Previous reports of autoantibodies against enolase, aldolase and pyruvate kinase

Auto-antibodies	**Disease**	**Detail**	**Reference**
Anti-alpha enolase	Cancer associated retinopathy	*In vitro* retinal cell apoptosis	Adamus G et al., 1998
Anti-alpha enolase	Lymphocytic hypophysitis (pituitary endocrinopathy)	-	O'Dwyer DT et al., 2002
Anti-alpha enolase	Schizophrenia	Conformational epitope	Deckmann M et al., 2002
Anti-alpha enolase	Hashimoto's encephalopathy	-	Ochi H et al., 2002
Anti-NSE	Glaucoma	Low titres	Maruyama I et al., 2002
Anti-alpha enolase	Discoid lupus erythemasosus	-	Gitlits VM et al., 1997
Anti-alpha enolase	SLE, systemic sclerosis, mixed cryoglobulinaemia	Impaired plasminogen binding	Moscato S et al., 2000 Pratesi F et al., 2000
Anti-alpha enolase	Rheumatoid arthritis	Only 36 of 145 patients	Saulot V et al., 2002
Anti-Aldolase A	Rheumatoid arthritis	-	Ukaji F et al., 1999

Anti-alpha enolase	ANCA positive vasculitis (37%) SLE (10/41)	With active renal disease	Moodie FD et al., 1993
Anti-alpha enolase	Autoimmune polyglandular syndrome	-	Peterson P et al., 1996
Anti-alpha enolase	Behcet's disease	-	Lee KH et al., 2003
Anti-alpha enolase	Primary membranous and lupus nephropathy	-	Wakui H et al. 1999
Anti-alpha enolase	Primary sclerosing cholangitis	Low titres only	Orth T et al., 1998
Anti-alpha enolase	Ulcerative colitis (10%) Crohn's disease (18%)	-	Roozendaal C et al., 1998
Anti-alpha enolase	Endometriosis	-	Walter M et al., 1995
Anti-alpha enolase	Primary biliary cirrhosis	-	Akisawa N et al., 1997
Anti-alpha enolase	Rheumatic fever	-	Fontan PA et al., 2000

10.2.6. Striatal vulnerability

If the hypotheis that anti-neuronal glycolytic enzyme antibodies can induce extrapyramidal movement disorders is correct, the hypothesis should be able to explain why the basal ganglia (striatum) is particularly vulnerable to these antibodies. As yet, there has been no investigation into the regional expression of glycolytic enzymes on neuronal membranes. It is possible that some neurons express these glycolytic enzymes at higher levels and are therefore more vulnerable to attack. However, there is a large body of investigation that has demonstrated that different neuronal populations have different vulnerabilities to insults. Specifically, the basal ganglia is particularly vulnerable to hypoxia, hypoglycaemia and ischaemia (Calabresi P et al., 2000). Energy failure and mitochondrial respiratory failure has been proposed to be the most important cause of the selective vulnerability of the basal ganglia neurons in Huntington's disease (which presents with chorea) (Calabresi P et al., 2000). Interestingly the protein responsible for Huntington's disease (Huntingtin) has been shown to bind to, and possibly inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme in energy metabolism (Guyot MC et al., 1997). Likewise, the protein atropin, responsible for dentatorubral-pallidoluysian atrophy (another degenerative disorder presenting with extrapyramidal movement disorders and predominant basal ganglia degeneration) also binds to the key glycoytic enzyme GAPDH (Ferrante RJ et al., 1997). It has been shown that certain subpopulations in the striatum are particularly vulnerable to deficits in energy metabolism and glutamate-mediated excitotoxicity. In particular, medium spiny neurons (MSN) (also known as striatal GABAergic projecting cells) are highly vulnerable and precociously damaged in disorders affecting energy metabolism, whereas striatal cholinergic interneurons (large aspiny cells) are spared by these

insults (Mitchell IJ et al., 1999; Calabresi P et al., 2000). This selective vulnerability of striatal GABAergic neurons (MSN) may be partly related to their different membrane properties. Specifically MSN have a high resting membrane potential and are more hyperpolarized compared to aspiny neurons under resting conditions (Calabresi P et al., 2000). Hypoxia, hypoglycaemia and ischaemia cause membrane depolarisation of striatal spiny neurons (followed by activation of calcium channels, release of neurotransmittors and potential cell death), whereas cholinergic aspiny neurons are hyperpolarized and protected during energy failure (Calabresi P et al., 2000). This selective vulnerability of striatal GABAergic spiny neurons to energy failure may explain why antibodies binding to membrane glycolytic enzymes can cause the post-streptococcal CNS phenotypes. GABA is the primary inhibitory neurotransmitter in the CNS. Failure of the inhibitory striatal neurons may result in striatal excitation and subsequent extrapyramidal movements and psychiatric disorders.

10.2.7. The molecular mimicry hypothesis

For a molecular mimicry hypothesis to stand-up to scrutiny, it is necessary for the precipitating micro-organism to express the mimicking protein, preferably on the cell surface. Streptococcal organisms express the glycolytic enzymes enolase, aldolase and pyruvate kinase on the cell surface (Pancholi V, Fischetti VA, 1998; Buckley ND and Hamilton IR, 1994; Wilkins JC et al., 2003). Most investigation has been into streptococcal surface enolase (SEN). SEN is similar to membrane bound human enolase in its role as a plasminogen receptor, intimately involved in the ability of streptococci to adhere to and invade pharyngeal cells (Pancholi V et al., 2003). The Pancholi/Fischetti group have also shown that antibodies to streptococcal surface enolase also cross react with human alpha enolase, supporting a cross-reactive molecular mimicry hypothesis (Fontan PA et al., 2000). In support of the role of this

process in post-streptococcal sequealae, patients with acute rheumatic fever have higher levels of anti-SEN and anti-human alpha enolase antibodies compared to healthy controls and controls with uncomplicated Streptococcal pharyngitis (Fontan PA et al., 2000). The mechanism of cross-reactivity is not yet apparent. The streptococcal glycolytic enzymes share a high level of amino acid homology with the human glycolytic enzymes, however it is possible that the epitope is conformational rather than sequential.

10.2.8. Criticism of methodology and future directions

The methods employed in this thesis, and the results from this thesis could be criticised for the following reasons:

1. **Rat brain was employed rather than human brain.** Rat brain provided me with the best opportunity of purifying the candidate autoantigen, predominantly due to the supply of plentiful fresh rat brain tissue. The preceding experiments (comparing rat with human brain) reassured me that rat brain was a valid surrogate for human brain. Likewise, the use of recombinant human brain proteins and purified commercial human proteins, subsequently reassured me that the findings were valid.
2. **The human antibodies have only been shown to bind to the solubilsed (SDS treated) autoantigens, not the membrane autoantigens.** Indeed the Western blotting employed here may alter, expose or remove epitopes on the autoantigens therefore altering the protein. The work in this thesis has not shown that the autoantibodies bind to the membrane forms of these glycolytic enzymes. Further experiments are required using the neuronal membrane in its physiological state (such as immunohistochemistry using intact neurons) to

determine whether the autoantibodies bind to the membrane forms of these enzymes.

3. **The effects of autoantibodies on neuronal function have only used commercial polyclonal antibodies.** Further experiments should purify patient IgG (preferably only the autoantibodies that bind to autoantigens). Purified human IgG should be used in the neuronal cell culture experiments measuring apoptosis. Further experiments could further determine potential functional effects of antibodies, such as measuring effects on membrane energy metabolism, intracellular energy metabolism and effects on neurotransmission and cell signalling.
4. **The sensitivity and specificity of these anti-neuronal glycolytic enzyme autoantibodies (anti-NGE Ab) needs to be determined in larger patient and control cohorts.** Developing a standardised ELISA may best improve confidence in the sensitivity and specificity. An ELISA would also allow opportunity to examine the potential role of these antibodies in neuropsychiatric syndromes such as Tourette syndrome and obsessive-compulsive disorder.
5. **The molecular mimicry hypothesis has only been theoretically examined.** To properly examine a possible cross-reactive autoantibody response between the streptococcal epitopes and neuronal epitopes, patient IgG against the neuronal autoantigens needs to be purified. This IgG could then be used against streptococcal epitopes to examine antibody cross-reactivity (either using synthesised candidate streptococcal epitopes or using random peptide libraries). Streptococcal organisms grown from post-streptococcal autoimmune neuropsychiatric patients should be used rather than random

streptococcal organisms. Molecular mimicry at the T-cell level should also be undertaken.

6. **Other markers of autoantibody pathogenicity have not been performed.** Purified human anti- NGE Ab needs to be used in an animal model (passive transfer of antibodies) to further examine antibody pathogenicity. In addition, purified neuronal glycolytic enzyme antigens should be immunized into animal models to determine whether these antigens produce CNS disease. Likewise purified Streptococcal glycolytic enzymes should also be immunised into animal models to determine pathogenicity. Finally, in order to further demonstrate autoantibody pathogencitiy, patients with defined anti-NGE Ab should be plasmapheresed to determine whether removal of these antibodies results in clinical improvements.
7. **Are other autoantigens relevant in post-streptococcal autoimmune neuropsychiatric disorders?** The crude antigen use in this thesis is likely to contain mainly soluble cellular extracts. Membrane constituents can sometimes require specific purification strategies. It is possible that autoantibodies against other membrane constituents (other than the neuronal glycolytic enzymes) are relevant in post-streptococcal neuropsychiatric disorders. Likewise, it is possible that other neuronal glycolytic enzymes may be autoantigens. For example glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an immunogenic cell surface streptococal protein with a neuronal membrane analogue. GAPDH has been shown to form complexes with enolase, aldolase, pyruvate kinase and NaK ATPase in neuronal membrane channels.
8. **How can autoantibodies against ubiquitous neuronal glycolytic enzymes produce a relatively specific neurological syndrome?** The autoantigens

defined in this thesis are (predominantly) neuronal isoforms. However, previous regional examination of these glycolytic enzymes has failed to demonstrate enrichment of these proteins in the basal ganglia. Indeed, these enzymes are relatively equally distributed in the cortex, deep grey matter and cerebellum. For these autoantibodies to be pathogenic, there must be other explanations for these antibodies to produce a relatively specific basal ganglia syndrome. Possible explanations include:

- The regional distribution of these glycolytic enzymes on the membrane has not been examined. Regions with high-energy requirements (such as the basal ganglia) may express more glycolytic enzymes on the neuronal membranes.
- The basal ganglia may be more vulnerable than other brain regions to the metabolic insult induced by these autoantibodies.
- As previously mentioned, pathological studies have shown that the inflammatory infiltrate is not purely localised to the basla ganglia, but involves other brain regions including the cortex.

9. **Are these autoantibodies specific to post-streptococcal CNS disorders?**

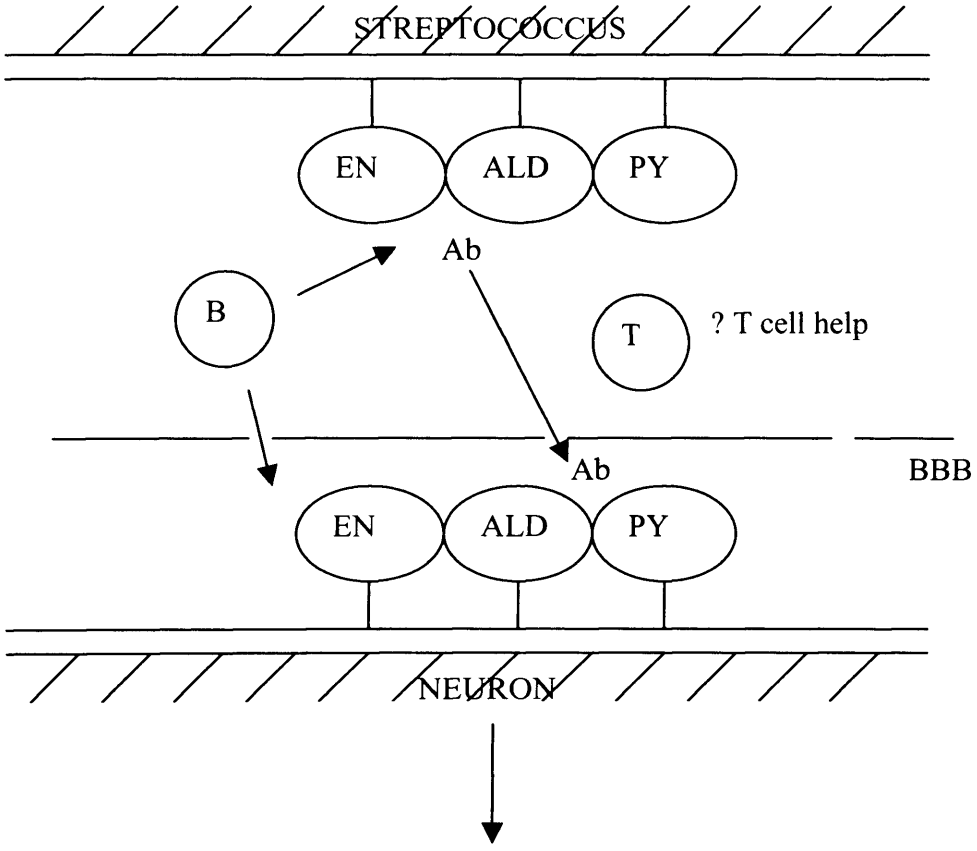
The previous reports of anti-enolase antibodies in a number of autoimmune and immune mediated disorders raise doubt on to the specificity of the antibodies. However the majority of the autoantibodies reported in this thesis are to neuronal isoforms of glycolytic enzymes enriched in, or specific to, the CNS. The possibility that these antibodies are an epiphenomenona or are secondary to tissue damage remains possible.

Chapter 11. Conclusions

The conclusions of this thesis are as follows:

1. The spectrum of post-streptococcal neuropsychiatric disorders is broader than previously described and includes extrapyramidal movement disorders (chorea, tics, dystonia and Parkinsonism). Psychiatric disorders commonly co-exist with the movement disorders, particularly emotional disorders such as obsessive-compulsive disorder and anxiety.
2. There appears to be a genetic vulnerability involved in disease expression. Likewise, the patient sex and age influence phenotypic expression. Pre-existing neuropsychiatric disorders such as attention deficit hyperactivity disorder may predispose children to the development of movement disorders after post-streptococcal autoimmune induction.
3. Magnetic resonance neuroimaging is typically normal, although inflammatory changes with particular involvement of the basal ganglia may occur. These inflammatory changes were most common in the dystonic or Parkinsonian patients.
4. Patients with post-streptococcal autoimmune neuropsychiatric disorders have autoantibodies against neuronal glycolytic enzymes. These neuronal autoantigens are expressed on the neuronal membrane and have previously been shown to be involved in energy metabolism, cell signalling and support neurotransmission. Provisional investigation suggests that autoantibodies against these targets can have pathogenic effects on neurons. These immunological conclusions are summarised in Figure 11.1.

Figure 11.1. Summary of immunological conclusions form this thesis.



Key:
EN: enolase
ALD: Aldolase
PY: Pyruvate kinase
BBB: Blood brain barrier
B: B cell
T: T cell
Ab: antibody

Chapter 12. References

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Chapter 13. Appendices

APPENDIX 1. Clinical characteristics of post-streptococcal dyskinesias and associated psychiatric morbidity.

Age of movement disorder onset (years) and sex	Movement disorder	ICD-10 psychiatric disorders			Movement disorder course (disease duration in years)
		Emotional	Conduct	Hyperkinetic	
1.2m	Dystonia, chorea	-	-	-	Relapsing remitting (1.8)
1.3 f	chorea	-	-	-	Persistent (1)
1.5m	chorea	OCD	-	Hyperkinesis (oth.)	Persistent (13)
2m	tics	OCD, social phobia, depression	Conduct (other)	Hyperkinesis	Persistent (13)
2f	Chorea, dystonia	-	-	-	Resolution (0.25)
2m	Myoclonus, dystonia	Panic attacks, gen. anxiety	Conduct (other)	-	Relapsing remitting (5)
3m	tics	-	-	-	Relapsing remitting (4)
3m	tics	Sep. anxiety, gen. anxiety, OCD	ODD	-	Relapsing remitting (5)
3f	Stereotypies	OCD	ODD	-	Persistent (7)
4m	Dystonia	-	-	-	Resolution (0.2)
4f	chorea	-	-	-	Persistent (0.5)
5m	tics	Sep. anxiety, gen. anxiety, OCD	-	Hyperkinesis	Persistent (5)
5f	tics	Trichotillomania, depression	Conduct (other)	-	Persistent (2)
6m	tics	OCD	-	-	Persistent (1)
6m	tics	-	-	-	Relapsing remitting (3)
7f	tics	OCD	-	-	Persistent (8)
7m	Chorea, tremor	-	-	-	Relapsing remitting (7)
7f	Tics, stereotypies	OCD	-	-	Persistent (2)
7f	chorea	-	-	-	Persistent (0.5)
7m	tics	-	-	Hyperkinesis	Relapsing remitting (2)
7m	chorea	-	-	-	Persistent (2)

Age and sex	Movement disorder	ICD-10 psychiatric disorders			Movement disorder course (disease duration in years)
		Emotional	Conduct	Hyperkinetic	
8f	chorea	-	ODD	-	Relapsing remitting (1.5)
8f	chorea	Gen. anxiety	-	Hyperkinesis	Resolution (0.25)
8m	tics	-	-	-	Relapsing remitting (2)
8m	tics	-	Conduct (other)	-	Relapsing remitting (7)
8m	tics	OCD	-	-	Persistent (4)
8f	tics	-	-	-	Relapsing remitting (1)
8m	Dystonia, chorea	Gen. anxiety, spec. phobia, depression	-	-	Relapsing remitting (1.5)
10m	chorea	Gen. anxiety	ODD	Hyperkinesis	Persistent (0.25)
10f	chorea	Sep. anxiety, gen. anxiety, OCD, social phobia, depression	ODD	-	Relapsing remitting (1.5)
10f	Chorea, tremor, opsoclonus	Gen. anxiety	-	-	Resolution (0.2)
11m	chorea	-	-	-	Resolution (0.8)
11f	chorea	-	-	-	Resolution (0.35)
11f	chorea	Depression	-	-	Resolution (0.8)
12f	chorea	-	Conduct (other)	-	Resolution (0.25)
12f	tics	-	-	-	Resolution (0.2)
13m	tics	Gen. anxiety, specific phobia, OCD, depression	-	-	Relapsing remitting (2.5)
13f	chorea	Gen. anxiety, specific phobia, depression	ODD	-	Resolution (0.8)
14m	chorea	-	-	-	Resolution (0.25)
16f	Tremor, opsoclonus	-	-	-	Persistent (0.8)

APPENDIX 2. Clinical findings in poststreptococcal acute disseminated encephalomyelitis

Patient no.	Age (yr/gender)	Extrapyramidal movement	Behavioural change	Other neurology	Outcome (follow-up)
1	3/M	Axial and limb cogwheel rigidity, dystonic posturing, rest tremor	Emotional lability, inappropriate laughter	Confusion and lethargy, reduced conscious ness (GCS 7/15), meningism	Normal (2 yr)
2	4/M	Axial and limb rigidity, dystonic posturing	Inappropriate laughter, confusion	Reduced consciousness (GCS 5/15), quadriplegia	Normal (2.5 yr)
3	4/M	Axial and limb rigidity, rest tremor	Inappropriate behaviour, pallilalia and echolalia, attention deficit	Irritability and lethargy, reduced consciousness (GCS 11/15), quadriplegia	Obsessive-compulsive disorder (3 yr)
4	4/M	Paroxysmal hemidystonia	Inappropriate speech	Tonic clonic convulsion (2), somnolence, ataxic gait	Normal (2 yr)
5	5/M	-	-	Hemiplegia	Normal (1 yr)
6	6/M	Left hemidystonia	Emotional lability, separation anxiety	-	Left hemidystonia (6mo)
7	7/M	-	Emotional lability, apathy	Somnolence, bilateral optic neuritis	Relapse (2mo), normal (2.5 yr)
8	10/M	-	Confusion and disorientation, inappropriate speech	Reduced consciousness (GCS 6/15), transverse myelitis, autonomic dysfunction	Normal (5 yr)
9	13/F	-	-	Hemiplegia	Normal (1 yr)
10	14/F	-	-	Hemiplegia, tremor	Relapse (13 mo), normal (18 mo)

APPENDIX 3. Patient demographics and clinical characteristics of encephalitis lethargica-like patients

Age	Sleep disorder	Lethargy	Parkinsonism	Dyskinesia	Psychiatric	Other features
2f	hypersomnolence	absent	bradykinesia, rigidity, rest tremor	-	-	-
4m	hypersomnolence	present	bradykinesia, rigidity, rest tremor	-	emotional lability	encephalopathy, meningism
5m	hypersomnolence	present	bradykinesia, rigidity	-	Mutism	ophthalmoplegia, ptosis, nocturnal bradycardia
7f	sleep inversion	present	bradykinesia	dystonic jaw	mutism, anxiety, depression, apathy	hyperventilation
8m	insomnia	absent	bradykinesia, postural instability	generalised dystonia	mutism, agitation, poor social interaction	encephalopathy, seizures
9f	sleep inversion	absent	bradykinesia, rigidity	oculogyric crises, hemiballismus	mutism, disinhibition, compulsive touching	ophthalmoplegia, hiccough
10f	hypersomnolence	present	bradykinesia	-	aggression, depression, apathy	-
10m	hypersomnolence	absent	bradykinesia	motor tics	mutism, depression	-
11f	insomnia	absent	rest tremor	facial grimacing	catatonia, agitation, panic attacks	seizures
13f	hypersomnolence	present	bradykinesia, postural instability	-	Apathy	ophthalmoplegia, meningism, optic neuritis
13m	hypersomnolence	absent	bradykinesia	generalised dystonia	mutism, confusion	encephalopathy, memory loss
14f	hypersomnolence	present	akinesia	Blepharospasm	mutism, depression, auditory hallucinations	pupillary light abnormality
15f	hypersomnolence	present	bradykinesia, rest tremor	dystonic posturing	Depression	memory loss
15m	sleep inversion	absent	bradykinesia, rest tremor	oculogyric crises, dystonia	mutism, catatonia, compulsions	-
15m	hypersomnolence	present	bradykinesia, rigidity, rest tremor	oculogyric crises	anxiety, paranoia	pupillary light abnormality, hiccough
16m	-	absent	bradykinesia, rigidity, rest tremor	-	-	hyperventilation
17m	sleep inversion	absent	bradykinesia	motor and vocal tics, stereotypies	mutism, catatonia, OCD, trichillomania	hyperventilation
35m	hypersomnolence	present	bradykinesia, rigidity, rest tremor	-	OCD, depression	ophthalmoplegia
69m	sleep inversion	present	bradykinesia, rigidity, rest tremor	Stereotypies	mutism, apathy, confusion	encephalopathy, pinpoint pupils
69f	hypersomnolence	absent	rigidity, rest tremor	dystonia, chorea	-	encephalopathy, memory loss, seizures

APPENDIX 4. Negative or normal investigations in encephalitis lethargica patien

Investigations were tailored according to the patient age, presentation and course. (number in brackets)

Aetiology	Diagnostic investigation
Infectious	CSF herpes simplex virus PCR (n= 12), CSF enterovirus pcr (n=5), CSF EBV PCR (n=8), CSF measles antibody (n=4), CSF bacterial culture (n= Lyme serology (n=6), Whipples, varicella zoster, influenza, arbovirus, pi adenovirus, parvovirus, bartonella, HIV (all n=2)
Systemic autoimmune disease	Anti-nuclear antibody* (n=16), complement (n=5), angiotensin convertii antibodies (n=3)
Metabolic	CSF lactate (n=14), creatinine kinase (n=4), ammonia (n=4), plasma ami acids (n=8), very long chain fatty acids (n=4), white cell enzymes (n=6), acanthocytes (n=6)
Biochemical	Copper (n=12), caeruloplasmin (n=9), thyroid function tests (n=10), porp metabolism (n=16), urine toxicology (n=7),
Genetic	Huntington's disease (n=3), DRPLA (n=3), SCA-3 (n=2), DYT-1 (n=4)

*Anti-nuclear antibody was ≤1:80 in all patients.

APPENDIX 5. Buffers, chemicals, solutions, standards and hardware

Chromatography: Buffers

Buffer	Uses	Constituents
Sodium phosphate buffer, pH 7.0	Binding buffer in chromatography	500ml double distilled water 0.87g $Na_2 HPO_4$ 0.47g $Na H_2 PO_4$
Citric acid buffer, pH 3.0	Elution or binding buffer in chromatography	500ml double distilled water 8.61g Citric acid 2.65g Sodium citrate
Tris-HCl buffer, pH 8.0	Binding buffer in chromatography	500ml double distilled water 1.2g Tris 4ml 1 Molar HCl

Chromatography: Hardware

Hardware	Uses	Product number
AKTA Fluid phase liquid chromatography	Fluid phase liquid chromatography	AKTA Pump P-920 Monitor UPC-900 Fraction Collector Frac-900/901 Compaq deskpro computer. All Amersham Pharmacia Biotech
UNICORN software for FPLC	Software for FPLC computer	Version 3.20, Amersham Pharmacia Biotech
HiTrap Q FF, 1ml	Ion exchanger, anion	Amersham Biosciences, 17-5053-01
HiTrap Q FF, 5ml	Ion exchanger, anion	Amersham Biosciences, 17-5156-01
HiTrap SP FF, 1ml	Ion exchanger, cation	Amersham Biosciences, 17-5054-01
HiTrap SP FF, 5ml	Ion exchanger, cation	Amersham Biosciences, 17-5157-01
HiTrap desalting, 5ml	Desalting, changing the buffer	Amersham Biosciences, 17-1408-01

HIC Selection kit, 1ml	Hydrophobic interaction selection kit	Amersham Biosciences, 17-1349-01
HiTrap rProtein A FF, 1ml	IgG purification	17-5079-02
HiTrap Butyl FF, 5ml	Hydrophobic interaction	17-5197-01

Chromatography: Chemicals

Product	**Company and log. number**
Sodium phosphate, monobasic, anhydrous $Na\ H_2\ PO_4$	Sigma S-0751
Sodium phosphate, dibasic, anhydrous $Na_2\ HPO_4$	Sigma S-7907
Citric acid, monohydrate, $C_6\ H_8\ O_7.\ H_2O$	Sigma C-1909
Citric acid, trisodium salt dihydrate $C_6\ H_5\ O_7\ Na_3.\ 2H_2O$	Sigma C-3434
Tris(hydroxymethyl)aminomethane, $C_4\ H_{11}\ NO_3$	Sigma T-6791
NaCl	Sigma S-9625
Hydrochloric acid, HCl, 1 Molar	BDH, 190686W

PAGE, electrophoresis and ELISA: Buffers and developing solutions

Buffer	**Uses**	**Constituents, product number**
MOPS SDS Running buffer (Invitrogen)	Running buffer in PAGE	500ml (20×) 3-(N-morpholino) propane sulphonic acid 104.6g (1M) Tris base 60.6g (1M) SDS 10.0g (69.3mM) EDTA 3.0g (20.5mM) Ultrapure water 500ml

MES SDS running buffer (Invitrogenn)	Running buffer in PAGE	500ml (20×) 2-(N-morpholino) ethane sulphonic acid 97.6g (1M) Tris base 60.6g (1M) SDS 10.0g (69.3mM) EDTA 3.0g (20.5mM) Ultrapure water 500ml
NuPAGE LDS sample buffer (4×)	Sample solubilisation for PAGE	Invitrogen, NP0007
Colormetric developing solution	Western blotting	4-Chloro-1-Naphthol 20µg Hydrogen peroxide 50µl Methanol 20ml Acetate buffer 5ml Distilled water 75ml
Enhanced chemiluminescent developing solution	Western blotting	Supersignal West Pico Chemiluminescent substrate, Pierce kit, 34080
IEF pH 3-10 sample buffer (2×)	IEF buffer before isoelectric focussing	Invitrogen, LC5311
IEF Anode (lower) buffer (50×)	IEF anode	Invitrogen, LC5300
IEF Cathode (upper) (50×)	IEF cathode	Invitrogen, LC5370
Acetate buffer	For colormetric development solution	136g Sodium acetate trihydrate 22.5g glacial acetic acid 5 litres distilled water For working strength, dilute 1:10
0.05Molar Carbonate buffer	Binding antigen to ELISA plate	0.5M: 1 litre of distilled water with 13.85g Na_2CO_3 and 26.1g $NaHCO_3$. working strength must be diluted 1:10.
Detector solution for ELISA	Detection of antibody reactivity	11ml of acetate buffer (working strength), 20µg OPD, 11µl Hydrogen peroxide.
TCA fixation buffer	Fixing IEF gels	In 1000ml double distilled water, 100g TCA 10g SSA

PAGE, electrophoresis and ELISA: Hardware

Product	Product number
Pre-cast NuPAGE 4-12% Bis-Tris gels, 2D (10 gels)	Invitrogen, NP0326BOX
Pre-cast NuPAGE 4-12% Bis-Tris gels, 10 well (10 gels)	Invitrogen, NP0321BOX
Pre-cast NuPAGE 10% Bis-Tris gels, 9 well (10 gels)	Invitrogen, NP0307BOX
Pre-cast IEF gels, pH 3-10, 10 wells (10 gels)	Invitrogen, EC6655A
PowerEase 500 electrophoresis tank	Invitrogen, E18700EU
Trans-Blot transfer medium, cut to 9cm square (nitrocellulose)	Bio-Rad 162-0094
Nunc-immuno Plate, Maxisorp surface	Nunc brand products

PAGE, electrophoresis and ELISA: Chemicals and solutions

Product	Company and log. Number
Colloidal blue staining kit- Stainer A	Invitrogen, product number 46-7015
Colloidal blue staining kit- Stainer B	Invitrogen, product number 46-7016
Silver staining kit	Amersham Biosciences, product number 17-1150-01
Molecular weight marker- SeeBlue plus 2	Invitrogen, product number LC5925
Ethyl Alcohol, C_2H_5OH	Hayman Ltd. UN1170
Methanol, CH_3OH	Hayman Ltd. UN1230
Acetic Acid	BDH, 27013BV
Coumassie R-250 0.1%	

Tri-Chloro-Acetic acid	Sigma, T-6399
Sulfosalicylic acid (SSA)	
Tween 20 (Polyoxyethylenesorbitan monolaurate)	Sigma P-1379
4-Chloro-1-Naphthol	Sigma C-8890
o-Phenylene-Diamine (OPD)	Sigma P-1526
Hydrogen peroxide (H_2O_2) 100 volumes	Fisher Chemicals H/1750/15
Bovine specific albumin (BSA)	Sigma A-7906

Proteomics and protein purification: Buffers

Product	**Company**
Tissue Protein extraction reagent (T-PER)	Pierce, USA (no. 78510)
Protease inhibitor cocktail for mammalian tissues	Sigma (P 8340)

Proteomics and protein purification: Hardware

Product	**Company**
Millipore microcentrifuge	Microcon YM-10, 42406

Proteomics and protein purification: Chemicals

Chemical	**Company, product number**
Ammonium sulphate, $(NH_4)_2 SO_4$	Sigma A-2939
DL-Dithriothreitol (DDT)	Sigma D-0632
Biuret protein measurement, Reagent A	Bio-Rad Dc Protein assay, 500-0113
Biuret protein measurement, Reagent B	Bio-Rad Dc Protein assay, 500-0116

Commercial Antibodies and antigens

Product	Company, source
Human neuron-specific enolase (NSE), *(500μg)*	Advanced Immunochemical incorporated (via Insight Biotechnology limited), purified using chromatography,
Human non-neuronal enolase (NNE), *(50μg)*	Accurate chemical and scientific corporation, Westbury, New York. Purified using chromography from brain.
Enolase from Baker's yeast, yeast (9mg solid, ~6.5mg protein)	Sigma-Aldrich, purified from S. cerevisiae, E6126
Enolase from rabbit muscle, (5.5mg protein/ml)	Sigma-Aldrich, purified from rabbit muscle, E0379
Plasminogen from human plasma, lyophilised powder, 6-9 units/mg protein	Sigma-Aldrich, purified from human plasma, P5661
Goat anti-human plasminogen HRP conjugated, 10mg/ml	Abcam, ab7336-1
Goat anti-pyruvate kinase, 10μg/μl	Abcam, ab6191
Goat anti-enolase C-19, 200μg/ml	Santa Cruz Biotechnology, sc-7455
Goat anti-aldolase, 10 mg/ml	Abcam, ab6190-100
Rabbit anti-human IgG, HRP conjugated	Dako, P0406
Rabbit anti-goat Immunoglobulins, HRP conjugated	Dako, P0160

APPENDIX 6. Publications

1. **Dale RC**, Church AJ, Cardoso F, Goddard E, Cox TC, Chong WK, Williams A, Klein NJ, Neville BG, Thompson EJ, Giovannoni G. Poststreptococcal acute disseminated encephalomyelitis with basal ganglia involvement and auto-reactive antibasal ganglia antibodies. Ann Neurol. 2001 Nov;50(5):588-95.
2. **Dale RC**, Church AJ, Surtees RAH, Lees AJ, Adcock JE, Harding B, Neville BGR, Giovannoni G. Encephalitis lethargica syndrome: 20 new cases and evidence of basal ganglia autoimmunity. Brain. 2004 Jan;127(Pt 1):21-33.
3. **Dale RC**, Heyman I, Surtees RAH, Church AJ, Giovannoni G, Goodman R, Neville BGR. Dyskinesias and associated psychiatric disorders following streptococcal infections. Arch Dis Child 2004; 89: 604-610.
4. **Dale RC**, Church AJ, Surtees RA, Thompson EJ, Giovannoni G, Neville BG. Post-streptococcal autoimmune neuropsychiatric disease presenting as paroxysmal dystonic choreoathetosis. Mov Disord. 2002 Jul;17(4):817-20.
5. **Dale RC**, Church AJ, Benton S, Surtees RA, Lees A, Thompson EJ, Giovannoni G, Neville BG. Post-streptococcal autoimmune dystonia with isolated bilateral striatal necrosis. Dev Med Child Neurol. 2002 Jul;44(7):485-9.
6. **Dale RC**. Autoimmunity and the basal ganglia: new insights into old diseases. QJM. 2003 Mar;96(3):183-91. Review.
7. **Dale RC**, Heyman I. Post-streptococcal autoimmune psychiatric and movement disorders in children. Br J Psychiatry. 2002 Sep;181:188-90. Review.
8. **Dale RC**. [Streptococcus pyogenes and the brain: living with the enemy] Rev Neurol. 2003 Jul 1-15;37(1):92-7. Spanish. Review.
9. **Dale RC**, Candler PM, Church AJ, Wait R, Pocock JM, Giovannoni G. Neuronal surface glycolytic enzymes are autoantigen targets in post-streptococcal autoimmune CNS disease. J Neuroimmunol. 2005 Dec 11; [Epub ahead of print]